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EFFECTS OF THE ANTIDEPRESSANT/ANTIPANIC DRUG PHENELZINE AND AN N-ACETYL ANALOGUE ON BIOGENIC AMINES AND AMINO ACIDS

by

KEVIN F. McKENNA ©

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND
RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
IN
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KF McKenna

11711 - 35 Avenue
Edmonton, Alberta
T6J 2W9

DATED: April 19th, 1995

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled EFFECTS OF THE ANTIDEPRESSANT/ANTIPANIC DRUG PHENELZINE AND AN N-ACETYL ANALOGUE ON BIOGENIC AMINES AND AMINO ACIDS by KEVIN F. McKENNA in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Medical Sciences.

M.T. Martin-Iverson (Chair)

Glen B. Baker

G.B. Baker (Supervisor)

R T Coutts

R.T. Coutts (Cosupervisor)

J.I. Goldberg

A.J. Greenshaw

W. G. Dewhurst

W.G. Dewhurst

S.H. Kennedy (External Examiner)

DATED: April 11, 1995

ABSTRACT

The antidepressant/antipanic drug phenelzine (PLZ) is a monoamine oxidase (MAO) inhibitor which also elevates brain levels of γ-aminobutyric acid (GABA). Its analogue, N^2-acetyl-PLZ (N^2AcPLZ), has been shown to have relatively strong MAO-inhibiting properties and has been proposed as a potential antidepressant drug.

This thesis reports the results of comprehensive experiments on the effects of PLZ and N^2AcPLZ on some neurotransmitter amines and the amino acids GABA and alanine (ALA) in brain and on the metabolism of PLZ. N^2AcPLZ was a good inhibitor of MAO-A and -B *ex vivo* and *in vitro*; like PLZ it produced a significant elevation of biogenic amines and reduction in the levels of their metabolites in rat brain, after both acute and chronic administration. Both PLZ and N^2AcPLZ caused a down-regulation of α_2-adrenoceptors, a common characteristic of antidepressants.

PLZ, but not N^2AcPLZ , produced a marked elevation of brain levels of GABA and ALA. Chronic (28-day) administration of PLZ produced small, but significant, decreases in activity of GABA- and ALA-transaminase in brain.

The GABA- and ALA-elevating effects of PLZ were reversed by pretreatment with the MAO inhibitors tranylcypromine and pargyline, further supporting the proposal that such actions of PLZ are due in large part to a metabolite formed by the action of MAO directly on PLZ.

Chronic administration of PLZ and N^2AcPLZ resulted in no changes in the density or affinity of $GABA_A$ receptors or benzodiazepine receptors in rat cortex and hippocampus.

Indirect and direct evidence was provided for the formation of *p*-hydroxyphenelzine (*p*-OH-PLZ) as a metabolite of PLZ. Uptake and release experiments in brain showed that *p*-OH-PLZ differed considerably from PLZ in ability to alter uptake and release of biogenic amines.

After 2 and 4 weeks of PLZ administration to psychiatric patients, there were dramatic increases in levels of biogenic amines and, in general, decreases in levels

of their metabolites in 24 hour urine samples. However, levels of 5-hydroxindole-3-acetic acid, phenylacetic acid and *p*-hydroxyphenylacetic acid (acid metabolites of 5-HT, β-phenylethylamine and *p*-tyramine) did not change significantly from pretreatment values.

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ABBREVIATIONS

ALA	alanine
ALA-T	alanine transaminase
GABA	γ-aminobutyric acid
GABA-T	γ-aminobutyric acid transaminase
AMI	amitriptyline
ANOVA	analysis of variance
APA	American Psychiatric Association
m-CPP	*m*-chlorophenylpiperazine
CGI-I	Clinical Global Impression of Improvement Scale
COMT	catechol 0-methyl transferase
d	day
DA	dopamine
DCC	dicyclohexylcarbodiimide
DEHPA	di-(2-ethylhexyl)phosphate
DIG	distilled in glass
DMI	desmethylimipramine
DOPAC	3,4-dihydroxyphenylacetic acid
ECT	electroconvulsive therapy
EDTA	ethylendiamine tetraacetate disodium salt
FA	fast acetylator
g	gram
GAD	glutamic acid decarboxylase
GC	gas chromatography
GC-MS	gas chromatography/mass spectrometry
GLY	glycine
h	hour
HPLC-EC	high pressure liquid chromatography-electrochemical detection
HVA	homovanillic acid

5-HIAA	5-hydroxyindole-3-acetic acid
p-OH-PLZ	*para*-hydroxyphenelzine
p-OHPAA	*para*-hydroxyphenylacetic acid
5-HT	5-hydroxytryptamine
IMI	imipramine
ip	intraperitoneal
IPR	iprindole
ILEU	isoleucine
kg	kilogram
LEU	leucine
3MT	3-methoxytyramine
MAO	monoamine oxidase
MAOI	monoamine oxidase inhibitor
MHPG	3-methoxy-4-hydroxyphenyl glycol
MPP$^+$	1-methyl-4-phenyl pyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
N^{2}AcPLZ	N^2-acetylphenelzine
NA	noradrenaline
NAD	nicotinamide adenosine dinucleotide
NME	normetanephrine
NOR	nortriptyline
NSD	no significant difference
OCD	obsessive compulsive disorder
PD	panic disorder
PAA	phenylacetic acid
PEA	β-phenylethylamine
PFBC	pentafluorobenzoyl chloride
PFP-OH	pentafluorophenol
PLZ	phenelzine (2-phenylethylhydrazine)
PTSD	posttraumatic stress disorder

REM	rapid eye movement
SA	slow acetylator
SEM	standard error of the mean
SIQ	semi-interquartile range
SSRIs	selective serotonin reuptake inhibitors
T	tryptamine
T_3	triiodothyronine
T_4	tetraiodothyronine
TA	tyramine
p-TA	*para*-tyramine
TCA	tricyclic antidepressant
TCP	tranylcypromine
TMI	trimipramine
TRP	tryptophan
VAL	valine
VEH	vehicle
VMA	vanillylmandelic acid

INTRODUCTION

A. GENERAL INTRODUCTION

Monoamine oxidase inhibitors (MAOIs) are a class of drugs which share the ability to inhibit the enzymes monoamine oxidase-A and/or -B (MAO-A, MAO-B), but vary widely in other important pharmacokinetic and pharmacodynamic properties. The MAOIs have found an important niche in clinical psychiatry where they are used as a key component in the multimodal therapy and rehabilitation of persons suffering from serious psychiatric illness. Patients afflicted with a wide variety of conditions may potentially benefit from an MAOI; in particular, those with affective (mood) or anxiety disorders often respond positively following several weeks of treatment (Nutt and Glue, 1989, 1991).

Phenelzine (2-phenylethylhydrazine, PLZ), an irreversible inhibitor of MAO-A and MAO-B, is a popular MAOI in clinical use at present. Despite the fact that PLZ has been utilized since 1959, there are serious gaps in our knowledge of the metabolism and neurochemical effects of this drug (Baker and Coutts, 1989). Advancing our understanding of how the MAOIs exert beneficial and deleterious effects allows the development of more specific and safer treatment approaches. A glimmer of light may also be shed on the underlying causes and pathophysiology of the affective and anxiety disorders. The neurotransmitters of greatest interest with regard to the etiology and pharmacotherapy of affective/anxiety disorders are noradrenaline (NA), dopamine (DA), 5-hydroxytryptamine (5-HT) and γ-aminobutyric acid (GABA), and these have been the focus of the studies described in this thesis.

In elucidating mechanisms of therapeutic effect, awareness of the metabolic routes of a compound is an important but often ignored factor. Many psychotropic agents have biologically active metabolites with unique properties that may contribute to the clinical effect of the parent drug (Potter and Manji, 1990; Young, 1991; Caccia and Garattini, 1992; Baker *et al.*, 1994). Genetic polymorphisms of drug-metabolizing enzymes give rise to distinct subpopulations of patients that differ in their ability to metabolize medications (Evans and White, 1964; Brosen and Gram, 1989; Coutts, 1994). For many years PLZ was considered to be metabolized by acetylation, a metabolic process known to be under genetic control (Evans and White, 1964; Baker and Coutts, 1989).

One of the possible acetylated metabolites of PLZ with biologic activity is N-2-acetylphenelzine (N^2AcPLZ). Studies by Coutts *et al.* (1990, 1991) have indicated that N^2AcPLZ is a very minor metabolite of PLZ but that it retains MAO-inhibiting properties and should be considered as a potential antidepressant drug in its own right. As little research has been done to date examining the neurochemical properties of N^2AcPLZ, a number of the studies described in this thesis have compared the effects of PLZ and N^2AcPLZ following acute and chronic administration in rodents. Similarities and differences in effects on neurotransmitter levels, enzyme activity, and neurotransmitter receptor function and density may then be related to the unique structures of these compounds.

Oxidative pathways of metabolism are also under genetic control (Brosen and Gram, 1989) and now appear to be more important in the biotransformation of PLZ than is acetylation (Robinson *et al.*, 1985). The possibility of ring hydroxylation as a metabolic route for PLZ in rodents and human subjects was examined in this thesis. The formation of such a hydroxylated metabolite could not only contribute to the pharmacokinetics and MAO-inhibiting profile of PLZ, but could affect the ability of this drug to alter the uptake and release of neurotransmitter amines. Consequently, the effects of *para*-hydroxy-PLZ (*p*-OH-PLZ) on uptake and release of NA, DA and 5-HT were examined.

A clinical project was also conducted in which a group of psychiatric patients suffering from affective or anxiety disorders were administered PLZ and urinary excretion of a number of biogenic amines and their metabolites were measured at 2 and 4 weeks after commencement of therapy with PLZ.

B. ANTIDEPRESSANT DRUGS

B.1 Affective and Anxiety Disorders

The affective and anxiety disorders are among the most common conditions diagnosed in clinical psychiatry (Weissman and Boyd, 1982; Myers *et al.*, 1984; Bland *et al.*, 1988a,b; Spaner *et al.*, 1994). While some patients present with a pure affective or anxiety disorder, these disorders often coexist and overlap with other serious psychiatric illnesses such as schizophrenia, anorexia nervosa, bulimia and alcohol or drug abuse. A mixture of several disorders is often seen in the same patient (Torgersen, 1990; Thompson *et al.*, 1989; Wittchen and Essau, 1989; Breier *et al.*, 1985; Wetzler and Katz, 1989) and treatments are often effective for more than one disorder, suggesting fundamental connectivity rather than discontinuity. It can be difficult to study "pure disorders" because of this pervasive overlap.

Of the classification schemes (nosology) that have been proposed in psychiatry over the years, the most popular in North America currently is the Diagnostic and Statistical Manual of the APA (American Psychiatric Association, 1994); volume 4 of this manual is now available and is commonly called DSM-IV. This classification system allows multiple diagnoses and is atheoretical with regard to the etiology or origin of mental disorders. Diagnoses are made based on the symptoms experienced by the patient and the behaviour of the patient as observed by the clinician. This allows for a reliable system in which clinicians are likely to come to similar diagnoses but may be flawed by not reflecting underlying biological causes and pathophysiology. It is important to investigate the potential causes (etiology) of a disorder in addition to making the diagnosis.

The affective or mood disorders in DSM-IV include bipolar disorder, depressive disorder, cyclothymia, and dysthymia. In bipolar disorder (previously called manic depressive illness) and cyclothymia, the mood will periodically swing from euthymia (normal mood) to depression or mania. Cyclothymia refers to a milder variant where the mood swings are attenuated, causing less dysfunction and disruption of the patient's life. The essence of these disorders is the manic episode, in which the patient experiences a distinct period of an expansive, elevated mood, accompanied by increased self-esteem (grandiosity) and energy, a decreased need to sleep, the subjective sense that thoughts are racing, excess talkativeness, distractibility and an increase in the time spent in goal-directed, pleasurable and often dangerous activities. The cornerstones in the pharmacologic management of bipolar disorders are mood-stabilizing drugs which include lithium salts and the anticonvulsants valproic acid, carbamazepine and clonazepam (Ballenger and Post, 1980; Prien and Gelenberg, 1989; Jefferson, 1990; Post, 1990). Exposure to an antidepressant in the absence of a mood stabilizer may precipitate mania, an event that confirms the diagnosis of bipolar disorder.

Depressive and dysthymic disorders are approximately ten times more common than bipolar disorder (Myers *et al.*, 1984; Weissman and Boyd, 1982), with reported incidences in the general population of approximately 5% or greater (Kaplan *et al.*, 1994; Spaner *et al.*, 1994). Depressed patients experience a sustained sadness that is not solely in reaction to adverse circumstances, an inability to experience pleasure (anhedonia), a sense of hopelessness that may lead to suicidal ideation or intent, diminished energy, impaired concentration and changes in appetite and sleep patterns (Table 1). Dysthymia is a mild form of depressive illness which never meets the full criteria for a major depression and also tends to be chronic in nature. Dysthymic patients often describe themselves as having been unhappy for as long as they can remember in contrast to the more episodic and discrete nature of major depressive episodes. When depression is left untreated, the mortality rate due to suicide has been reported to be a staggering 15%, a rate 30 times that in the general population (Robins and Guze, 1970). The recurrence rate of depression is as high as 75-80%, leading to chronic disruption of the patient's life (Zis and Goodwin, 1979).

Misunderstandings may occur when discussing depressive illness due to the large number of meanings ascribed to the word depression. In medicine, the word is taken to mean a serious pervasive disorder that is distinctly different from normal and is not solely a reaction to unpleasant circumstances. Grief is an experience similar in phenomenology to depression but considered a normal reaction to loss. If grief becomes severe, protracted and causes significant dysfunction it would eventually be considered a depression (Zisook and Shuchter, 1991). Like the anxiety disorders, depression is a syndrome, a group of illnesses which share

Table 1: Diagnostic criteria for major depression (adapted from DSM-IV, APA).

At least five of the following symptoms have been present during the same two-week period and represent a change from previous functioning; at least one of the symptoms is either (1) depressed mood, or (2) loss of interest or pleasure.

1. Depressed mood.
2. Markedly diminished interest or pleasure (anhedonia).
3. Significant weight loss or weight gain.
4. Insomnia or hypersomnia.
5. Psychomotor agitation or retardation.
6. Fatigue.
7. Feelings of worthlessness or excessive or inappropriate guilt.
8. Diminished ability to think or concentrate, or indecisiveness.
9. Recurrent thoughts of death, recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide.

common symptoms and signs. In all likelihood, patients arrive at the common syndrome through varying paths. For some, a genetic predisposition is paramount, whereas in others emotional trauma, loss, medical disorders or the use (or abuse) of drugs may be causative factors. In most cases a number of factors may be necessary to arrive at the endstate, and their relative importance remains a matter of conjecture. Regardless of the etiology, it appears that 70% of patients with depression will respond following several weeks of continual antidepressant therapy (Morris and Beck, 1974; Baldessarini, 1985).

The anxiety disorders include panic disorder [PD] (Table 2), generalized anxiety disorder (Table 3), obsessive compulsive disorder (OCD), post-traumatic stress disorder (PTSD) and phobic disorders. Phobic disorders are common, often mild, and involve the avoidance of specific situations (snakes, heights, enclosure). In general, the treatment of phobic disorders involves exposure and desensitization to the feared stimulus. Two specific forms of phobia require comment due to their co-morbidity with PD. Agoraphobia, a fear of being in situations where help may not be available, is often seen in patients with a history of PD (Klein and Gorman, 1987; Klein and Klein, 1989; Pollack *et al.*, 1990). Common agoraphobic situations involve a fear of leaving home, a fear of being alone, or a fear of being in a place where help is not readily available. Social phobia, an avoidance of public situations where the patient may become the object of scrutiny or observation, is often seen in patients with PD and responds to treatment with MAOIs (Liebowitz *et al.*, 1985a). PTSD is a heterogenous group of disorders in which the patient has disturbing symptoms of depression and anxiety following a devastating event such as war, rape or natural disaster. OCD is a serious chronic disorder in which obsessions or

Table 2: Diagnostic criteria for panic disorder (PD) [adapted from DSM-IV, APA).

Recurrent, unexpected panic attacks (discrete periods of intense fear or discomfort) have occurred that lead to apprehension about having further attacks. At least four of the following symptoms occur abruptly during a panic attack and peak within 10 min:

1. shortness of breath (dyspnea) or smothering sensations.
2. dizziness, unsteady feeling, or faintness.
3. palpitations or accelerated heart rate (tachycardia).
4. trembling or shaking.
5. sweating.
6. choking.
7. nausea or abdominal distress.
8. depersonalization or derealization.
9. numbness or tingling sensations (paresthesias).
10. flushes (hot flashes) or chills.
11. chest pain or discomfort.
12. fear of dying.
13. fear of going crazy or of doing something uncontrolled.

Table 3: Diagnostic criteria for generalized anxiety disorder (adapted from DSM-IV, APA).

- Unrealistic or excessive anxiety and worry (apprehensive expectation), for a period of 6 months or longer

- Difficult to control the worry.

- Three of the following:
  (1) restless, keyed up
  (2) easily fatigued
  (3) difficulty concentrating
  (4) irritability
  (5) muscle tension
  (6) sleep disturbance

compulsions are experienced to a degree that they interfere with normal functioning and is most amenable to behavioural treatment and antidepressants that enhance serotoninergic function (Greist, 1990; Jenike, 1990; Boyer *et al.*, 1991). These drugs include tricyclic and novel antidepressants that block the neuronal uptake pump for serotonin (5-HT; 5-hydroxytryptamine) [Perse *et al.*, 1987; Jenike *et al.*, 1989a,b; Clomipramine Collaborative Study Group, 1991; Boyer, 1992; Freeman *et al.*, 1994; Lydiard, 1994].

The most commonly observed anxiety disorders are generalized anxiety disorder and PD. These disorders share common symptoms but the time course is precipitous and time-limited in PD and sustained or unrelenting in generalized anxiety disorder. The essential feature of PD is the panic attack, characterized by the sudden onset of apprehension and fear, and associated with autonomic and cardiorespiratory symptoms (Table 2). It follows a crescendo pattern, typically peaks in minutes and quickly dissipates. These attacks tend to fluctuate and recur. The marked distress that a panic attack creates often provokes the patient to seek medical attention. PD is often complicated by the development of anticipatory anxiety and phobic avoidance including agoraphobia (Klein and Klein, 1989; Lelliot *et al.*, 1989; Thompson *et al.*, 1989; Buller *et al.*, 1991). There is an extensive overlap between PD, generalized anxiety disorder, phobic disorders, depression and alcoholism (Weissman and Merikangas 1986; Thompson *et al.*, 1989; Kushner *et al.*, 1990; Wittchen *et al.*, 1991; Keller, 1992; Dick *et al.*, 1994). The benzodiazepines (particularly alprazolam), which enhance GABAergic function (Section F) and many antidepressants have been found useful in the treatment of generalized anxiety disorder and PD (Baldessarini, 1985; Liebowitz, 1989;

Dubovsky, 1990; Boyer 1992; Bakish *et al.*, 1993; Ballenger, 1993; Delafuente, 1993; Jonas and Cohon, 1993; Rosenberg, 1993; Shelton *et al.*, 1993; Johnson *et al.*, 1994). The splitting of PD from generalized anxiety disorder diagnostically has been controversial as they share common symptoms and treatment. The idea that generalized anxiety disorder is not amenable to treatment with antidepressants and PD does not respond to benzodiazepines has been proven wrong many times over the past decades in well controlled studies (Johnstone *et al.*, 1980; Charney *et al.*, 1986; Kahn *et al.*, 1986; Ballenger *et al.*, 1988; Noyes *et al.*, 1988; Lesser *et al.*, 1992; Schweizer *et al.*, 1993). The advantage of the benzodiazepines in the treatment of anxiety is their safety and immediate onset of clinical effect. The antidepressants will require several weeks administration prior to onset of effectiveness but are more useful when a mixture of disorders including depression is present.

B.2 Classes of Antidepressants

Until the 1950s, physical treatments for serious depression were limited to insulin-induced hypoglycemia and convulsive therapies induced by camphor or electric current (electroconvulsive therapy, ECT) [Baldessarini, 1985]. Of these, only ECT has continued to be utilized in the management of severe depression (APA Task Force on ECT, 1978; Yudofsky, 1982; Thompson *et al.*, 1994). While the psychotropic effects of naturally occurring substances have been recognized and exploited since antiquity, it is only during the past half century that a large number of effective drugs has become readily available for the treatment of major mental disorders. These advances were initiated by a number of fortuitous discoveries, rather than the planned and rational development of drugs that would have specific biologic effects. Subsequently, attempts were made to alter compounds structurally in specific ways in order to increase the selectivity of their effects. Drugs found effective in the treatment of depression have been classified in a simple fashion into the (1) MAOIs, (2) tricyclic antidepressants (TCAs), and (3) novel (or atypical) antidepressants.

The introduction of MAOIs as antidepressants in the late 1950's resulted from an attempt to develop antibiotics. In order to eradicate tuberculosis, scientists developed isonicotinic acid 2-isopropylhydrazide (iproniazid) [Figure 1], a derivative of the tuberculostatic isonicotinoylhydrazine (isoniazid). Iproniazid unexpectedly had a psychostimulant effect in tubercular patients, leading to behaviour that was described as "dancing in the hall" (Pletscher, 1991). Patients were noted to experience insomnia, increased energy and increased appetite. These observations led to clinical trials of iproniazid in chronically depressed, institutionalized patients, resulting in a 70% improvement rate (Crane 1957; Loomer *et al.*, 1957, 1958). Iproniazid was subsequently released and marketed as the first antidepressant, only to be recalled after being associated with a high incidence of hepatic dysfunction. The discoveries that isoniazid and iproniazid were inhibitors of the enzyme MAO (Zeller *et al.*, 1952a,b) and reversed the depressant effects of reserpine (Chessin *et al.*, 1956) suggested that inhibition of MAO might be important in producing the antidepressant response. This suggestion has been supported by the development of several clinically useful MAOIs, including the hydrazine compound phenelzine (PLZ) and the nonhydrazine tranylcypromine (TCP) [Figure 1]. A second generation

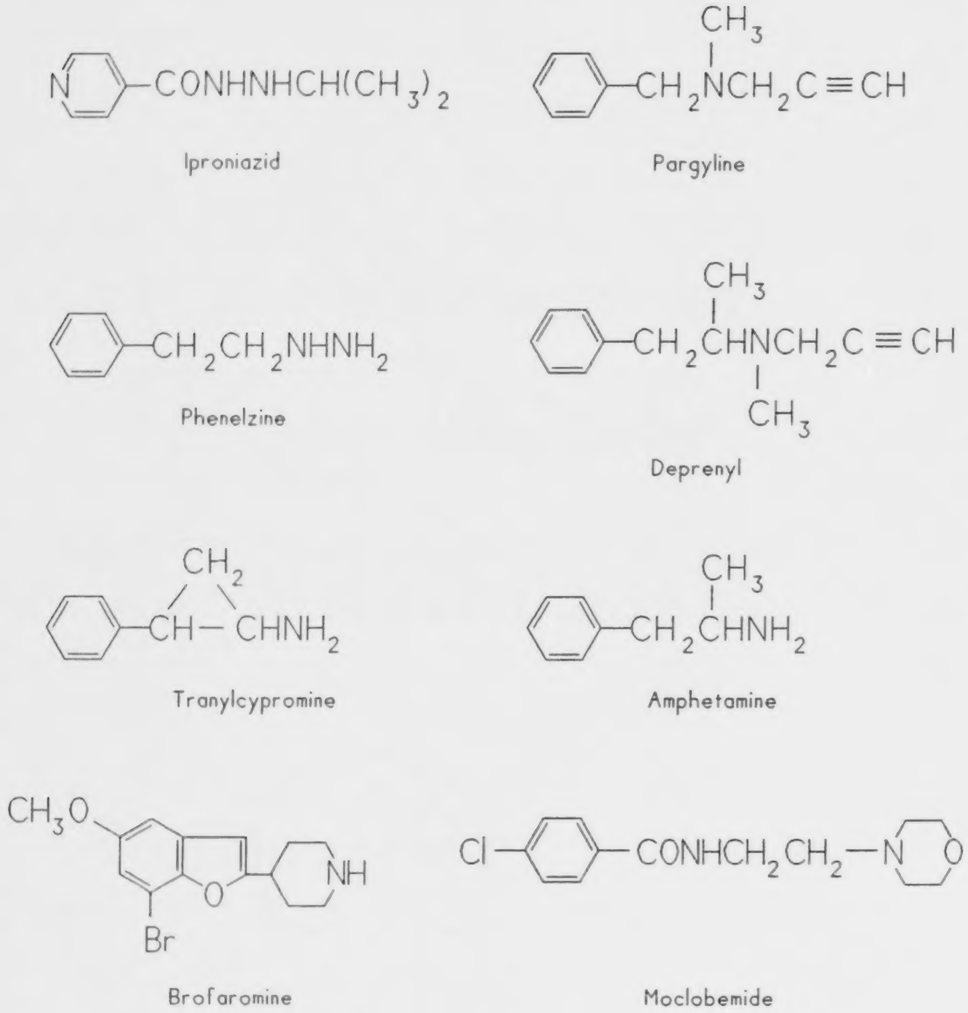
CONHNHCH(CH3)2
Iproniazid
CH3
CH2NCH2C≡CH
Pargyline
CH2CH2NHNH2
Phenelzine
CH3
CH2CHNCH2C≡CH
CH3
Deprenyl
CH2
CH—CHNH2
Tranylcypromine
CH3
CH2CHNH2
Amphetamine
CH3O
O
NH
Br
Brofaromine
Cl
CONHCH2CH2—N
O
Moclobemide

Figure 1: Structures of some monoamine oxidase inhibitors (MAOIs).

of MAOIs is currently being studied in order to develop safer and more specific compounds. Several of these such as (-)-deprenyl, brofaromine and moclobemide are now commercially available or in phase III clinical trials (Williams, 1984; Amrein *et al.*, 1989; Simpson and de Leon, 1989; Möller *et al.*, 1991; Heinze *et al.*, 1993; Paykel, 1993; Tiller, 1993; Chouinard *et al.*, 1994; Silverstone *et al.*, 1994; Verhoeven, 1994).

A parallel development in the late 1950's was the discovery that imipramine (IMI), a tricyclic compound, was efficacious in the treatment of serious depression (Kuhn, 1957). Most tricyclic antidepressants (TCAs) share a common structure consisting of two benzene rings joined by a central seven-member ring (Baldessarini, 1985). Numerous examples of this class are available, including desmethylimipramine (DMI), amitriptyline (AMI), nortriptyline (NOR), trimipramine (TMI), doxepin, protriptyline, and clomipramine (Figure 2). The TCAs possess a wide spectrum of biological activity but share the property of blocking the presynaptic uptake pump for the amines NA and/or 5-HT (with a much weaker effect on DA) [Hytell, 1982; Baker and Dewhurst, 1985]. In addition, most TCAs are antagonists of neurotransmitter receptors, including presynaptic and postsynaptic muscarinic (acetylcholine), histaminic, adrenergic and serotoninergic receptors (Baldessarini, 1985; Rudorfer and Potter, 1989). A knowledge of a TCA's antagonist potency for each type of receptor permits some insight into its propensity to cause side-effects related to receptor blockade. Common side-effects of TCAs related to blockade of receptors include dry mouth, blurred vision, urinary retention and constipation (muscarinic), sedation and weight gain (histamine) and postural hypotension (α_1-adrenergic) [Pollack and Rosenbaum, 1987]. The N-demethylated

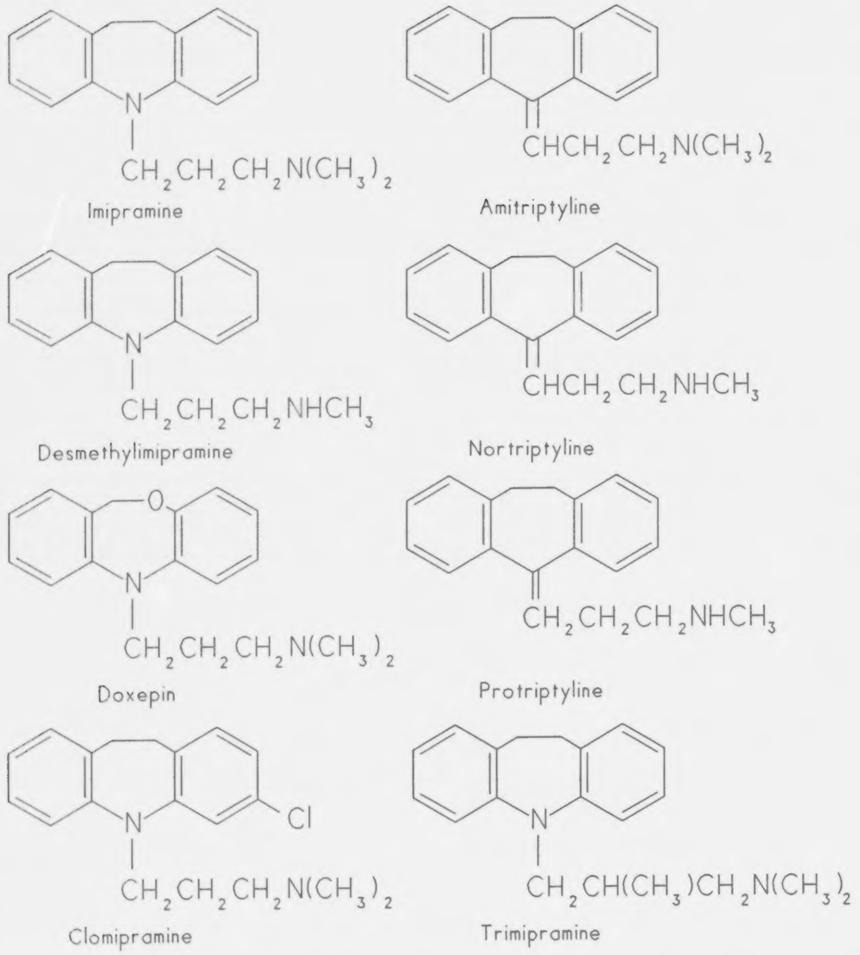
N
CH2CH2CH2N(CH3)2
Imipramine
CHCH2CH2N(CH3)2
Amitriptyline
N
CH2CH2CH2NHCH3
Desmethylimipramine
CHCH2CH2NHCH3
Nortriptyline
O
N
CH2CH2CH2N(CH3)2
Doxepin
CH2CH2CH2NHCH3
Protriptyline
N
Cl
CH2CH2CH2N(CH3)2
Clomipramine
N
CH2CH(CH3)CH2N(CH3)2
Trimipramine

Figure 2: Structures of tricyclic antidepressants (TCAs).

metabolites of IMI and AMI, DMI and NOR respectively, are more potent NA uptake inhibitors and possess less anticholinergic properties than the parent compounds. Because of the greater selectivity and decreased toxicity, DMI and NOR are popular in elderly and medically unstable patients, who might be intolerant of side-effects. Until the advent of the novel antidepressants, the TCAs were the most commonly used drugs for the treatment of depression. In addition, the TCAs are efficacious in the treatment of the anxiety disorders such as PD, OCD and generalized anxiety disorder (Liebowitz, 1989; Dubovsky, 1990).

Novel antidepressants represent drugs that do not fall readily into one of the other two classes. A number of drugs that selectively inhibit the uptake of NA, DA or 5-HT, but are not TCAs, are now available or in development. Selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine, fluvoxamine, sertraline and paroxetine are rapidly becoming popular and well tolerated treatments for depression (Asberg *et al.*, 1986; Fuller and Wong, 1989; Rudorfer and Potter, 1989; Boyer and Feighner, 1991; Richelson, 1994), PD (Liebowitz, 1989; Boyer, 1992; Bakish *et al.*, 1993; Sheehan *et al.*, 1993) and OCD (Jenike *et al.*, 1989b; Goodman *et al.*, 1990; Jenike, 1990; Boyer, 1992). Such agents have no anticholinergic or sedative properties but rather tend to be arousing and cause appetite suppression (Wernicke, 1985; Fuller and Wong, 1989; Leonard, 1993). Depressed patients with obesity find these agents useful in reducing weight as well as improving depressive symptomatology. Nefazadone selectively inhibits 5-HT uptake and antagonizes 5-HT_2 receptors (Eison *et al.*, 1990). It may reduce anxiety and improve sleep quality independent of sedation (Sharpley *et al.*, 1992; Fawcett, 1994). Trazodone, a triazolopyridine derivative, in addition to inhibiting 5-HT uptake, is a partial 5-HT agonist, and its metabolite, *m*-chlorophenylpiperazine (m-CPP), is a potent 5-HT agonist (Coccaro and Siever, 1985; Zohar *et al.*, 1987). Trazodone is commonly used in small doses at bedtime to induce sleep in patients treated with MAOI or SSRIs (Zimmer *et al.*, 1984; Nierenberg and Keck, 1989; Fabre, 1990). Selective NA and DA uptake inhibitors would be potentially useful, but progress in their development has been slow. Nonselective DA uptake inhibitors such as bupropion and nomifensine have been synthesized but are not available in Canada (van Scheylen *et al.*, 1977; Ferris *et al.*, 1982). Nomifensine, a tetrahydroisoquinoline agent which inhibits the uptake of both NA and DA, was found to be a useful antidepressant but because of an association with fatal hemolytic anemia was discontinued (Brogden *et al.*, 1979; Rudorfer and Potter, 1989). Bupropion only weakly inhibits the uptake of DA and its mechanism of action is controversial (Bryant *et al.*, 1983; Golden, 1988; Nemeroff, 1994). A relatively selective NA uptake inhibitor, maprotiline, has antidepressant effects but is also associated with an increased risk of seizures (Rudorfer *et al.*, 1984). Recent studies have reported a new class of antidepressants represented by venlafaxine (Schweizer *et al.*, 1991; Clerc *et al.*, 1993; Contú *et al.*, 1994) and duloxetine (Wong *et al.*, 1993) which are inhibitors of both NA and 5-HT reuptake but have a better side-effect profile than the TCAs (Wong *et al.*, 1993; Cunningham *et al.*, 1994).

Ligands of several NA and 5-HT receptor subtypes have therapeutic potential. Mianserin blocks presynaptic α_2-adrenergic inhibitory autoreceptors, leading to enhanced NA release into the synapse (Brogden *et al.*, 1978). Agonists of the 5-HT$_{1A}$-receptor, including buspirone, gepirone, ipsapirone and flesinoxan, have anxiolytic and antidepressant effects (Eison, 1990; Rickels, 1990; Robinson *et al.*, 1990; Asberg and Martesson, 1993; Deakin, 1993; Grof *et al.*, 1993). Amphetamine, which releases intracellular stores of NA, DA and, to a lesser extent, 5-HT may have antidepressant effects but, due to the potential for abuse, this treatment is usually reserved for specific situations (Warneke, 1990) such as treatment of refractory depressions or treatment of the elderly or of medically unstable patients with prominent anergia.

A number of agents such as iprindole and S-adenosylmethionine have been reported to have antidepressant efficacy with no effects on amine uptake (Lipinski *et al.*, 1984; Damlouji *et al.*, 1985; Baldessarini, 1985; Kagan *et al.*, 1990; Rosenbaum *et al.*, 1990; Bell *et al.*, 1994; Bressa, 1994). No mechanism of action has been established for iprindole, while S-adenosyl-methionine is an endogenous methyl donor. The triazolobenzodiazepines adinazolam and alprazolam have apparent antidepressant efficacy in addition to the anxiolytic effects they share with all the benzodiazepines (Rickels *et al.*, 1985, 1987; Eriksson *et al.*, 1987; Mooney *et al.*, 1988; Warner *et al.*, 1988; Kravitz *et al.*, 1990; Rimón *et al.*, 1991).

Numerous antidepressants are now available for clinical use. Most of these drugs have acute enhancing effects on synaptic NA and 5-HT levels, but some unusual agents are available. The antidepressants are now known to be effective in the treatment of a wide array of mental disorders and several could also be called antipanic or antianxiety drugs. The division of antidepressants into MAOIs, TCAs and atypical (novel) drugs was based on the history of their development and is now a confusing system of classification and outdated. The MAOIs share a common biologic effect while the TCAs share structure. Numerous novel agents block the uptake of biogenic amines and are similar in effect to the TCAs. A more useful classification would consider biologic effect, recognizing that many antidepressants, including PLZ, have multiple actions, each of which could be responsible for the remission of certain symptoms. Elucidation of the complete pharmacodynamic profile of a drug and its metabolites would facilitate our understanding of how these drugs work. The development of drugs with very selective effects would then greatly improve the specificity and safety of antidepressant therapy. The discovery of the antidepressant drugs has dramatically improved the care of patients and stimulated intensive research into the origins of mental illness, but the paucity of selective drugs has been a limiting factor.

B.3 Antidepressant Mechanisms of Action

Several hypotheses have been put forward to explain how antidepressants exert beneficial effects. Understanding antidepressant mechanisms of action may suggest which neurotransmitter systems are involved in the origin of mental illness. The biogenic amine hypothesis of depression was formulated following the serendipitous discovery that MAOIs and TCAs had therapeutic potential in the treatment of depression. Depression, it was hypothesized, resulted from a functional deficiency of NA and/or 5-HT at certain central synapses and antidepressants worked by overcoming this deficit (Bunney and Davis, 1965; Schildkraut, 1965; Lapin and Oxenkrug, 1969). This theory was also based on the observation that treatments which deplete (reserpine) or inhibit the synthesis (e.g. α-methyl-*p*-tyrosine) of the biogenic amines NA and 5-HT resulted in depression (Chessin *et al.*, 1956; Bunney and Davis, 1965; Schildkraut, 1965; Garver and

Davis, 1979). Antidepressants were thought to correct this deficit by elevating levels of the amine neurotransmitters through inhibition of their catabolism (MAOIs) or uptake mechanisms (TCAs, novel drugs). A refinement suggested that biochemically distinct subtypes of depression existed, each characterized by a dysfunctional neurotransmitter system. In some patients a disturbance in serotoninergic regulation existed, while in others noradrenergic mechanisms predominated (Maas *et al.*, 1972; Maas, 1978). If the neurochemical profile of a particular patient could be determined, a specific antidepressant could be prescribed and the chances of a recovery would be increased. Despite intensive research (Section C.2), such a hope has not yet been realized in daily clinical practice. The biogenic amine hypothesis of depression was an elegant and useful concept but several observations mitigated against such a simple mechanism completely explaining the clinical effects of the antidepressants. In particular, the requirement for several weeks of drug administration prior to symptom remission suggests that inhibition of MAO or blockade of the uptake mechanism, which occur shortly after drug intake, are only partial explanations.

Recent theories of antidepressant mechanisms of action have emphasized the delayed effects of elevated amine levels on presynaptic and postsynaptic receptor density and on second-messenger systems (reviews: Baker and Dewhurst, 1985; Heninger and Charney, 1987; Baker and Greenshaw, 1989; Hrdina, 1993; Nestler and Duman, 1995). Neurotransmitter receptors are integral membrane proteins to which neurotransmitters bind and, through various transduction mechanisms, elicit biologic effects. Several subtypes of receptor respond to each neurotransmitter, with each subtype differing in form and effect from the others. Ionotropic (receptor-mediated ion channel) and metabotropic (affecting enzymes and second messenger systems) receptors represent the two major families of receptors.

Changes in adrenergic and serotoninergic receptor sensitivity are observed following administration of many antidepressants, and the time-course of these changes approximates the onset of clinical recovery. The most commonly reported change is a decreased sensitivity of the β-adrenoreceptor-coupled adenylate cyclase system (Vetulani and Sulser, 1975; Vetulani *et al.*, 1976a,b; Sugrue, 1982; Maj *et al.*, 1984a; Sulser, 1987; Baker and Greenshaw, 1989). This observation has been relatively consistent among many antidepressants studied (including ECT), regardless of their primary biologic effect. It is likely that several mechanisms are involved in this heterologous desensitization, including shared second messenger systems and receptor cross-talk. Utilizing radioligand binding assays, a reduction in the density of β-adrenoreceptors has been reported in rat cortical tissue following chronic, but not acute, administration of MAOIs, TCAs, novel antidepressants and ECT (Banerjee *et al.*, 1977; Sellinger-Barnette *et al.*, 1980; Kellar *et al.*, 1981; Sugrue, 1983; Baker and Greenshaw, 1989). A notable exception is the SSRI group of drugs, where down-regulation of β-adrenergic receptors has only been observed at very high doses (Snyder and Peroutka, 1982; Wong *et al.*, 1985; Walmsley *et al.*, 1987; Byerley *et al.*, 1988; Goodnough and Baker, 1994).

Assessments of β-adrenoreceptor function using electrophysiologic experiments also reveal desensitization following chronic antidepressant treatment (Aghajanian, 1982; Schultz *et al.*, 1981). Finally, salbutamol, a β-receptor agonist (Brittain *et al.*, 1968), induces behavioral hypoactivity in rodents, an effect that is attenuated in animals treated chronically with antidepressants (Przegalinski, 1983, 1984). The effects of chronic antidepressant treatment on α-adrenergic receptors (α_1 and α_2) are less clear, but evidence supports enhanced sensitivity of α_1 receptors and diminished sensitivity of inhibitory α_2 autoreceptors (Svensson and Usdin, 1978; Cohen *et al.*, 1982a,b; Sugrue, 1983; Maj *et al.*, 1984a; Baker and Greenshaw, 1989). Clonidine, a selective α_2 agonist at low doses, suppresses rat locomotion, an effect that is attenuated following several weeks, but not days, of antidepressant treatment (Spyraki and Fibiger, 1980; Cohen *et al.*, 1982a; Greenshaw *et al.*, 1988). It has been suggested that changes in α_2-receptor density precede and may be responsible for the decrease in β-adrenoreceptors (Reisine *et al.*, 1982).

Serotoninergic receptors are also affected by antidepressants. Radioligand binding studies reveal a reduction in 5-HT_2 receptor density following chronic treatment with many antidepressants (Peroutka and Snyder, 1980; Snyder and Peroutka, 1984; Scott and Crews, 1986; Eison *et al.*, 1991) but not ECT (Kellar and Bergstrom, 1983). Surprisingly, chronic administration of SSRIs does not result in a down-regulation of 5-HT_2 receptors except at very high doses (Eison *et al.*, 1991; Lafaille *et al.*, 1991; Goodnough and Baker, 1994). Blier and de Montigny (1994) have proposed that chronic administration of the major classes of antidepressants results in a net increase in 5-HT neurotransmission, although the different types of antidepressants act through different mechanisms or combinations of mechanisms on 5-HT receptors, e.g. on responsiveness of somatodendritic 5-HT_{1A} autoreceptors, on function of terminal 5-HT autoreceptors, or on responsiveness of postsynaptic 5-HT receptors. Interactions between NA and 5-HT systems may be important in antidepressant action (Manier *et al.*, 1987; Sulser and Sanders-Bush, 1987), but this remains a controversial area of research, particularly with regard to possible mechanisms involved (Dumbrille-Ross and Tang, 1983; Helmeste, 1986; Asakura *et al.*, 1987; Aulakh *et al.*, 1988; Nowak and Przegalinski, 1988; Hensler *et al.*, 1991; Eiring *et al.*, 1992). Combinations of noradrenaline uptake inhibitors and 5-HT uptake inhibitors have been reported to be effective in treating depressed patients (Weilburg *et al.*, 1989; Nelson *et al.*, 1991; Seth *et al.*, 1992). As mentioned previously in this thesis, a new generation of drugs which are inhibitors of both NA and 5-HT reuptake appear to be promising antidepressants (Wong *et al.*, 1993; Cunningham *et al.*, 1994).

Several other neurotransmitters, including DA, acetylcholine, GABA and neuromodulating peptides, are apparently important in the etiology and treatment of depression. Some novel antidepressants (e.g. nomifensine and bupropion) have effects on DA, and it seems probable that DA is critical in certain aspects of depression such as anhedonia and psychomotor retardation (Fibiger, 1984; Davis 1989; Osman and Potter, 1991; Brown and Gershon, 1993; Papp *et al.*, 1994b). An enhancement of behavioural responses to DA agonists has been reported following administration of TCAs and MAOIs (Martin-Iverson *et al.,* 1983; Maj *et al.*, 1984b) and ECT has been reported to increase DA metabolite levels (Zis *et al.,* 1991). The effects of antidepressants on DA receptor functions are not clear, but several researchers have found evidence of down-regulation (desensitization) of the inhibitory presynaptic DA receptor following chronic antidepressant treatment (Antelman and Chiodi, 1981; Nielsen, 1986). Tanda *et al.* (1994) have recently proposed that stimulating DA neurotransmission in the prefrontal cortex may be a property shared by many antidepressants. The antidepressant effects of anticholinergic agents and the onset of severe depression in laboratory personnel accidently exposed to insecticides with anticholinesterase activity imply a role for acetylcholine in depression (Gershon and Shaw, 1961; Janowsky *et al.*, 1972; Janowsky and Risch, 1987). Many of the TCAs have anticholinergic activity which may be involved in their efficacy and side-effects (Maj *et al.*, 1984a). The importance of central nervous system peptides is suggested by the onset of psychiatric symptoms in patients with disorders of the thyroid and adrenal glands (Haskett and Rose, 1981). Steroid hormone administration is often complicated by depression (Kaufmann *et al.*, 1981; Ling *et al.*, 1981; Reckart and Eisandrath, 1990), and thyroid hormones (triiodothyronine [T3] and tetraiodothyronine [T4]) have some efficacy in the treatment of depression, particularly in patients already showing a partial response to an antidepressant (Joffe, 1990). A possible role for GABA in the etiology and treatment of depression and anxiety is emerging and is discussed in more detail in Section F.

The brain is a dynamic organ with multiple interacting neurotransmitter systems implicated in the etiology and treatment of depression. In all likelihood, a unitary mechanism of antidepressant action does not exist; antidepressants may potentially act through several neurotransmitter systems, with the bulk of evidence at present involving NA and 5-HT, but DA, acetylcholine, several peptides, and GABA also appear to be important.

C. MONOAMINE OXIDASE (MAO) AND INHIBITORS

C.1 Biology and Genetics of MAO

MAO is a flavin-containing enzyme found principally on outer mitochondrial membranes where it catalyzes the oxidative deamination of monoamines. Several of the biogenic monoamines, including NA, DA, 5-HT, tyramine (TA), tryptamine (T) and β-phenylethylamine (PEA) appear to be important in the etiology and treatment of affective/anxiety disorders (review: Baker and Dewhurst, 1985). Two discrete forms of the enzyme, MAO-A and -B, are distinguished by differences in substrate preference (Table 4), inhibitor specificity, tissue and cell distribution, immunological properties and amino acid sequence (Singer, 1987; Westlund *et al.*, 1988; Wells and Bjorksten, 1989; Weyler *et al.*, 1990). The presence of two distinct enzymes, originally suggested by the selective inhibition of MAO-A with clorgyline (Johnston, 1968) and MAO-B with L-deprenyl (Knoll and Magyar, 1972), has been confirmed in recent molecular genetic studies (Bach *et al.*, 1988; Hsu *et al.*, 1989; Grimsby *et al.*, 1990; Kanazawa, 1994). c-DNA clones of the two enzymes have been isolated and the nucleotide sequences determined (Bach *et al.*, 1988; Hsu *et al.*, 1989), confirming a high degree of sequence similarity (70%) and suggesting a common progenitor gene. The two forms of MAO differ in substrate preference (Table 4), but this specificity is relative rather than absolute.

The enzyme is expressed widely through eukaryotic organisms and in all mammals studied to date. Many cells express both forms of MAO, in differing proportions, but some tissues contain only one form of the enzyme, which has expediated purification of the enzymes. In human tissue, MAO-A is found in the placenta (Weyler and Salach, 1985) while MAO-B is found in platelets (Donnelly

Table 4: Substrates and inhibitors of MAO.

	MAO-A	MIXED	MAO-B
Substrates	adrenaline noradrenaline metanephrine serotonin (5-HT)	m,p-tyramine dopamine octopamine synephrine tryptamine N-methyltryptamine N,N-dimethyltryptamine	β-phenylethylamine phenylethanolamine o-tyramine benzylamine MPTP
Irreversible Inhibitors	clorgyline	phenelzine tranylcypromine isocarboxazid pargyline (more B) isoniazid iproniazid	(-)-deprenyl
Reversible Inhibitors	moclobemide brofaromine	amphetamine	

and Murphy, 1977). As platelets are relatively easy to procure, the vast majority of human research has examined MAO-B. The human brain expresses both enzymes but MAO-B predominates (80-95%) [Kalaria *et al.*, 1988], while in rat brain MAO-A predominates slightly over MAO-B. Immunohistochemical studies utilizing polyclonal or monoclonal antibodies in postmortem human brain have demonstrated that serotoninergic neurons contain predominantly MAO-B, while catecholaminergic neurons contain MAO-A (Levitt *et al.*, 1982; Westlund *et al.*, 1988). Both forms of MAO are present in glial and endothelial cells. These findings suggest that the two forms of MAO are independently regulated and perform different functions. The presence of MAO-B in serotoninergic neurons is a surprising finding as 5-HT is preferentially deaminated by MAO-A; MAO-B may function to degrade low levels of dietary and endogenous amines such as PEA or TA, having little effect on 5-HT unless levels of this amine are elevated. Under normal conditions the classical neurotransmitters metabolized by MAO (NA, DA, 5-HT) are preferentially stored in vesicles where they are not exposed to MAO. Free cytoplasmic and extraneuronal neurotransmitter amines would be most susceptible to MAO metabolism. The presence of high concentrations of MAO in the blood-gut and blood-brain barriers supports the idea that MAO serves a protective or detoxifying role (Blashchko, 1952; Murphy and Kalin, 1980).

In contrast, it has been suggested that MAO is responsible for the production of toxic oxidative metabolic products. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a contaminant of illicitly synthesized meperidine, was reported to cause an irreversible parkinsonian-like syndrome (Davis *et al.*, 1979; Langston *et al.*, 1983). The neurotoxicity is dependent on MPTP being converted into 1-methyl-4-phenyl-pyridinium (MPP^+) by vascular MAO-B. The MPP^+ is then sequestered in nerve terminals which selectively take up NA or DA, resulting in neuronal damage. Several researchers have proposed that oxidative products of MAO may lead to neuronal damage (Cohen, 1984; Lohr, 1991; Sandler *et al.*, 1993; Knoll, 1993). Hydrogen peroxide, formed in the course of the deamination reaction, may react with transition metals (Fe^{2+}, Cu^{2+}) to form highly reactive hydroxyl radicals (Lohr, 1991).

MAO-A and -B are encoded by separate genes located on the short arm of the X chromosome (Pintar *et al.*, 1981; Kochersperger *et al.*, 1986; Lan *et al.*, 1989). During development the MAO-A form is expressed first, followed by MAO-B, which increases in proportion through life. The complete absence of MAO and its corresponding mRNA has been described in a family of male cousins with a submicroscopic deletion of region Xp21-p11, an area including the Norrie disease gene (Sims *et al.*, 1989; Pettanati *et al.*, 1993). Norrie disease is a rare X-linked recessive neurologic disorder characterized by retinal dysplasia with blindness and, in 50% of patients, mental retardation and progressive hearing loss. Clinical features in these patients that may be related to complete absence of MAO include somatic growth failure, abnormal sexual maturation, autonomic nervous system dysfunction with hypotension, sleep disturbances with a marked reduction in the amount of rapid eye movement (REM) sleep, flushing, atonic seizures, motoric hyperactivity and hyperreflexia. Large reductions in the urinary excretion of the NA metabolites 3-methoxy-4-hydroxyphenyl glycol (MHPG) and vanillylmandelic acid (VMA) and the DA metabolite homovanillic acid (HVA) were observed in these individuals (Sims *et al.*, 1989). There was also a marked increase in the urinary excretion of PEA, a substrate of MAO-B. The neurochemical changes are dramatic and of a greater degree than seen in humans treated with MAOIs because in the case of MAOIs, complete inhibition of MAO would never be achieved.

Numerous attempts have been made to correlate hereditary variations in MAO activity with human disease. Large, as much as 50-fold, interindividual variations in platelet and fibroblast MAO activity have been described in studies of normal humans and those with psychiatric disorders (Murphy *et al.*, 1976; Breakefield *et al.*, 1981). Low MAO-B activity has been associated with stimulus seeking and suicidal behaviour (Donnelly *et al.*, 1979; Fowler *et al.*, 1980; von Knorring *et al.*, 1984), alcoholism (Sullivan *et al.*, 1979; von Knorring *et al.*, 1984) and bipolar affective disorder (Gershon *et al.*, 1979; Pandey *et al.*, 1980).

C.2 Biochemistry of MAO

MAO catalyzes an oxidative deamination of arylalkylamines, resulting in the formation of hydrogen peroxide and an aldehyde derivative of the amine (Figure 3). The aldehyde is then further reduced or oxidized to the corresponding alcohol or carboxylic acid respectively. Catechol O-methyl transferase (COMT) is also involved in the metabolism of the catecholamines, catalyzing the transfer of a methyl group to the OH group in the 3 position of the phenyl ring.

The catecholamine NA is metabolized by MAO and preferentially reduced to 3,4-dihydroxyphenylglycol. This is then converted to MHPG by COMT (Figure 4). NA may also be acted upon directly by COMT to form normetanephrine (NME) which is then converted by MAO to either MHPG or vanillylmandelic acid (VMA).

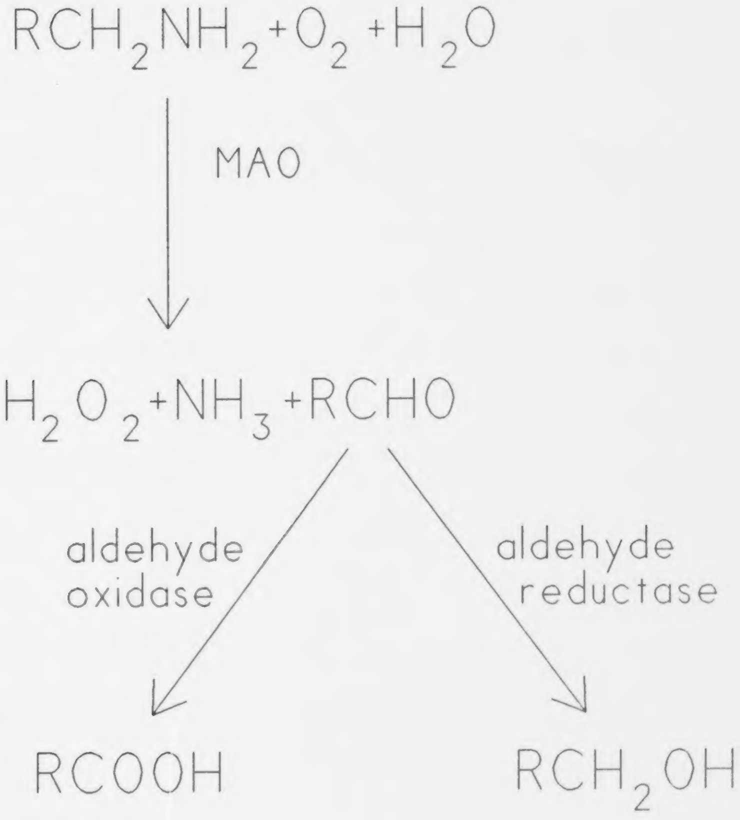
$RCH_2NH_2 + O_2 + H_2O$
MAO
$H_2O_2 + NH_3 + RCHO$
aldehyde oxidase
aldehyde reductase
RCOOH
RCH_2OH

Figure 3: The reaction catalyzed by MAO.

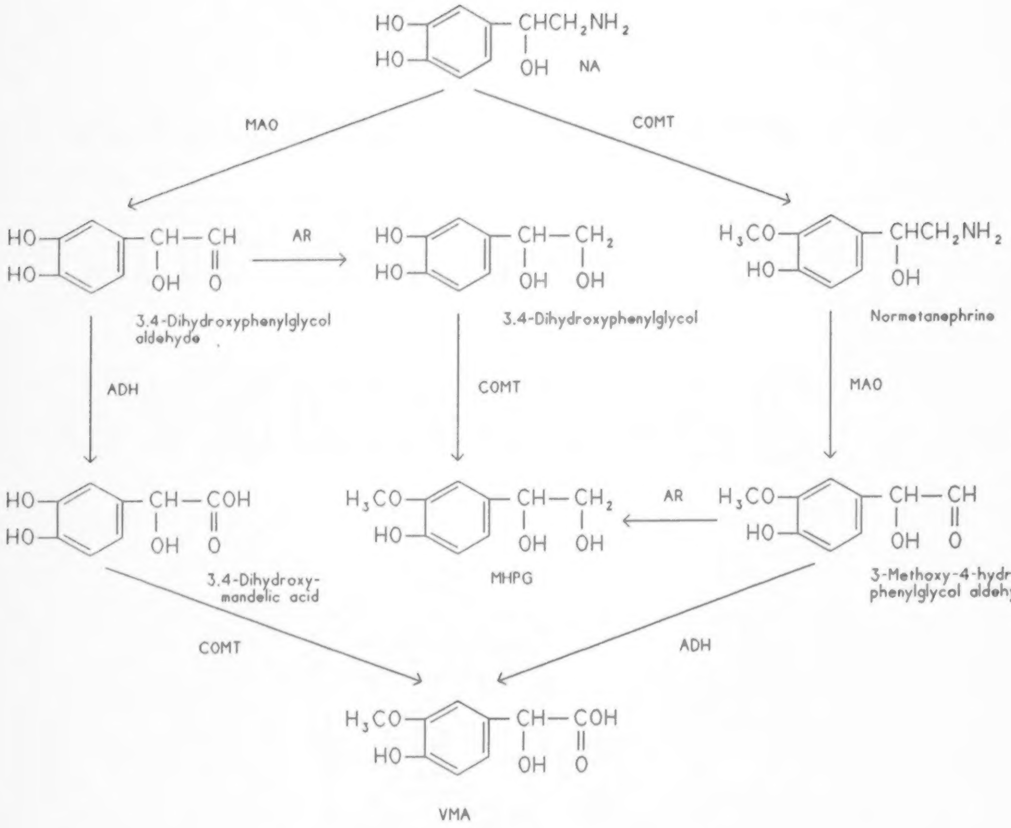
NA
MAO
COMT
AR
3.4-Dihydroxyphenylglycol aldehyde
3.4-Dihydroxyphenylglycol
Normetanephrine
ADH
COMT
MAO
AR
3.4-Dihydroxy-mandelic acid
MHPG
3-Methoxy-4-hydroxy-phenylglycol aldehyde
COMT
ADH
VMA

Figure 4: The catabolism of NA. MAO = monoamine oxidase; COMT = catechol O-methyltransferase; AR = aldehyde reductase; ADH = aldehyde dehydrogenase; VMA = vanillylmandelic acid.

Many studies have followed changes in urinary MHPG levels as a marker of NA metabolism and attempted to correlate MHPG levels with the diagnosis, severity of the condition or ultimate clinical response. One constraint in this type of research is the fact that peripheral sources of NA such as the adrenal gland produce a considerable amount of the total MHPG excreted. Theoretically, cerebrospinal fluid concentrations of MHPG should provide the best index of central NA turnover. Low pretreatment levels of urinary MHPG have been associated with a positive treatment response to IMI, which, because it is metabolized to DMI *in vivo*, is thought to primarily affect NA uptake (Maas *et al.*, 1972, 1982; Beckman and Goodwin, 1975; Garvey *et al.*, 1990). In studies utilizing PLZ, pretreatment MHPG levels had no predictive value (Beckman and Murphy, 1977; Sharma *et al.*, 1990). The evidence at present does not justify the use of pretreatment urinary MHPG levels as a predictive clinical tool, but further research in this area may be worthwhile in subgroups of depressed patients classified according to the presence of specific groups of symptoms.

Whereas the aldehyde produced in the metabolism of NA by MAO is reduced, in the case of DA it is mainly oxidized to 3,4-dihydroxyphenylacetic acid (DOPAC) [Figure 5]. COMT metabolizes DOPAC to HVA and DA directly to 3-methoxy-tyramine (3MT). Low CSF levels of HVA have been associated with subsequent suicide attempts and may be a marker of severe depression or depression associated with psychotic features (Montgomery and Montgomery, 1982; Agren, 1983; Roy *et al.*, 1989). DA is also crucially involved in psychotic illnesses such as schizophrenia and delusional disorder (Seeman, 1993).

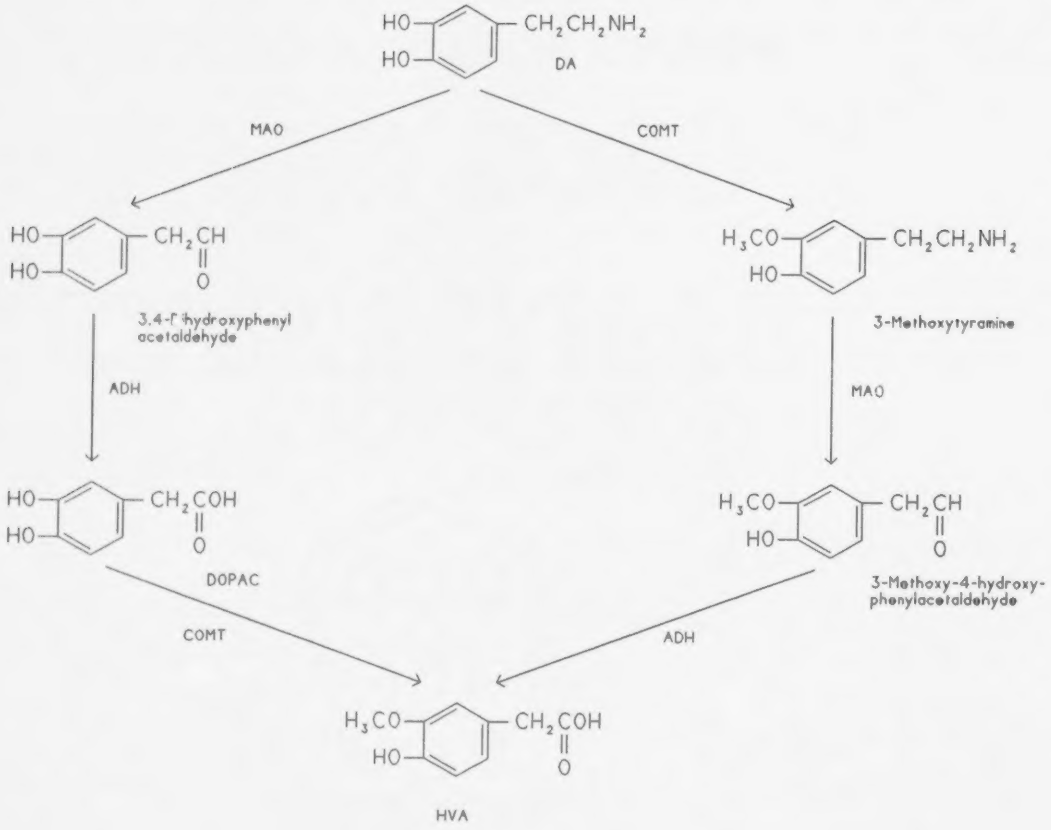
HO
HO
$CH_2CH_2NH_2$
DA
MAO
COMT
HO
HO
CH_2CH
O
3,4-Dihydroxyphenyl acetaldehyde
H_3CO
HO
$CH_2CH_2NH_2$
3-Methoxytyramine
ADH
MAO
HO
HO
CH_2COH
O
DOPAC
H_3CO
HO
CH_2CH
O
3-Methoxy-4-hydroxy-phenylacetaldehyde
COMT
ADH
H_3CO
HO
CH_2COH
O
HVA

Figure 5: The principal routes of catabolism of DA.

The major product formed in 5-HT catabolism in the brain is 5-hydroxyindole-3-acetic acid (5-HIAA) [Figure 6]. There is extensive literature concerning the use of 5-HIAA as a biological marker. Several studies have reported decreased levels of CSF 5-HIAA in depressed patients and an association between low CSF 5-HIAA and suicidal behaviour (Asberg *et al.*, 1976; van Praag, 1982; Agren, 1983; Asberg *et al.*, 1987). Several research groups have reported reductions in brain 5-HIAA and 5-HT in postmortem studies of suicide victims (Cheetham *et al.*, 1989; Stanley and Stanley, 1990). Although there is a growing body of evidence regarding this link between suicidal acts and low CSF 5-HIAA, it is becoming apparent that low levels of this metabolite may relate to aggressive behaviour or impulsivity in general (Traskman *et al.*, 1981; Banki *et al.*, 1984; Golden *et al.*, 1991).

C.3 MAO Inhibitors

Compounds of several distinct classes have been reported to be inhibitors of MAO. A widely accepted classification system categorizes MAOIs into irreversible versus reversible enzyme inhibitors and selective versus nonselective in their action on the two forms of MAO. The predominant MAOIs in clinical use for the past several years (PLZ and TCP) are irreversible and nonselective in their effects on MAO-A and -B, but selective irreversible [(-)-deprenyl (MAO-B)] and reversible inhibitors [moclobemide and brofaromine (MAO-A)] are now available commercially or undergoing clinical trials (Möller *et al.*, 1991; Elsworth and Roth, 1993; Tiller, 1993).

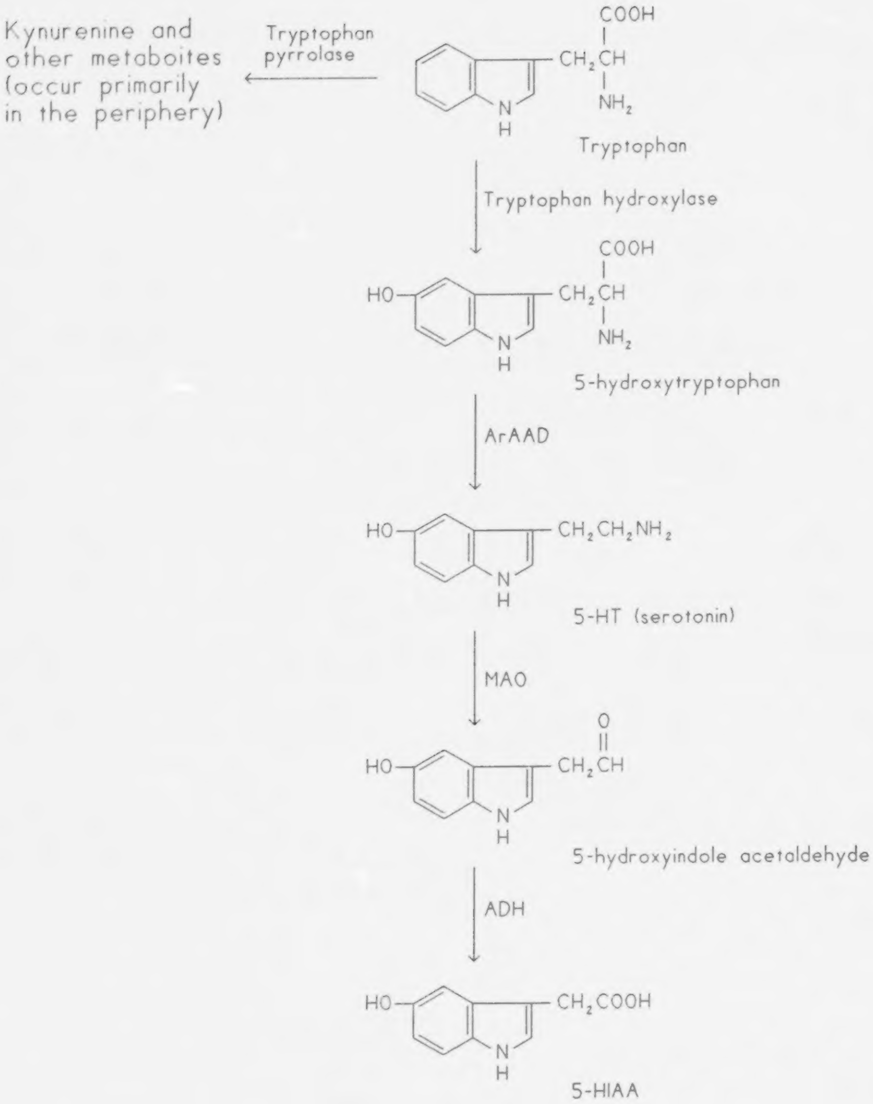
Kynurenine and other metaboites (occur primarily in the periphery)
Tryptophan pyrrolase
COOH
CH2CH
NH2
N H
Tryptophan
Tryptophan hydroxylase
HO
5-hydroxytryptophan
ArAAD
CH2CH2NH2
5-HT (serotonin)
MAO
O
5-hydroxyindole acetaldehyde
ADH
CH2COOH
5-HIAA

Figure 6: The principal routes of metabolism of 5-HT. ArADD = aromatic amino acid decarboxylase; MAO = monoamine oxidase; ADH = aldehyde dehydrogenase; 5-HIAA = 5-hydroxyindole-3-acetic acid.

MAOIs have been used in the treatment of tuberculosis (isoniazid and iproniazid), but are now used primarily in the pharmacotherapy of psychiatric and neurologic disorders. A wide spectrum of mood and anxiety disorders, including depression, atypical depression and panic disorder (Dowson, 1987; Murphy *et al.* 1987; Norman and Burrows, 1989; Nutt and Glue, 1989; Tyrer and Shawcross, 1988; Johnson *et al.*, 1994; Martin *et al.*, 1994) are amenable to pharmacotherapy with available MAOIs. The requirement for dietary restrictions to reduce TA consumption in patients receiving irreversible MAOIs has restricted their use among many psychiatrists and most general practitioners. The reversible selective MAO-A inhibitors, such as moclobemide and brofaromine, promise to be safer in this regard (Bieck *et al.*, 1989; Cooper, 1989; Möller *et al.*, 1991; Tiller, 1993) and may prove to be more popular than the older agents. (-)-Deprenyl, a selective MAO-B inhibitor, has not proven to be a particularly effective antidepressant (Elsworth and Roth, 1993), but it has been shown to attenuate the parkinsonian-like toxicity of MPTP (Heikkila *et al.*, 1984) and is also widely used in the treatment of idiopathic Parkinson's disease, both alone and in combination with L-DOPA (Birkmayer *et al.*, 1985; Tetrud and Langstrom, 1989; Parkinson Study Group, 1989, 1993). Birkmayer *et al.* (1985) reported that Parkinson patients treated with (-)-deprenyl in addition to L-DOPA live longer than those receiving L-DOPA without the (-)-deprenyl. It has also been claimed that (-)-deprenyl retards the progressive neuronal degeneration characteristic of Parkinson's disease (Tetrud and Langstrom, 1989; Parkinson Study Group, 1993). It has also been reported that (-)-deprenyl can act as a neuronal "rescue" agent (Tatton and Greenwood, 1991; Salo and Tatton, 1992) and that such effects may be unrelated to inhibition of MAO activity

(Ansari *et al.*, 1993). There are also reports of dramatically increased life-spans in rats treated chronically with (-)-deprenyl (Knoll, 1988; Milgram *et al.*, 1990), suggesting a longevity effect of this drug.

D. PHENELZINE (PLZ)

D.1 Clinical Efficacy

PLZ, a potent irreversible inhibitor of MAO-A and MAO-B, is used in a variety of psychiatric disorders including major depression (McGrath *et al.*, 1986; Janicak *et al.*, 1988), atypical depression (Paykel *et al.*, 1982a; Kayser *et al.*, 1985; Liebowitz *et al.*, 1985b, 1988a; Quitkin *et al.*, 1988, 1989, 1990), PD (Sheehan *et al.*, 1980; Buiges and Vallejo, 1987), bulimia (Walsh *et al.*, 1984), social phobia (Liebowitz *et al.*, 1985a, 1988b), bulimia nervosa (Walsh *et al.*, 1988) and post-traumatic stress disorder (PTSD [Davidson *et al.*, 1987; Kosten *et al.*, 1991]. PLZ has been reported to have advantageous properties in the treatment of depression associated with anxiety and to be slightly superior to other agents in the treatment of PD (Sheehan *et al.*, 1980; Nies, 1984; Tyrer and Shawcross, 1988; Quitkin *et al.*, 1990).

Several issues regarding the efficacy of PLZ require comment. For many years PLZ was reported to be useful mainly in the treatment of atypical depression (Sargant, 1961; Sargant and Dally, 1962; Stewart *et al.*, 1989; Quitkin *et al.*, 1990). The concept of atypical depression varied slightly but generally referred to a depression characterized by mood reactivity, extreme sensitivity to interpersonal loss or rejection, prominent anergia, increased appetite and increased sleep

(Quitkin *et al.*, 1990). This syndrome was known by a number of names including hysteroid dysphoria, neurotic depression, characterological depression and mixed anxiety-depression. Many patients with atypical depression also suffer from PD and anxiety symptoms. As several recent reports attest, PLZ is superior to IMI and AMI in the treatment of this subclass of depression (Quitkin *et al.*, 1989, 1990, 1994; Stewart *et al.*, 1989). PLZ has also been reported to be the treatment of choice for PD, being slightly superior to IMI in comparative trials (Sheehan *et al.*, 1980; Quitkin *et al.*, 1990). In the treatment of classical depressive disorders with the symptoms of insomnia, decreased appetite and loss of reactivity to the environment, PLZ appears to be equally efficacious to other antidepressants (McGrath *et al.*, 1986; Janicak *et al.*, 1988). The choice of treatment in this type of depression depends on a history of prior response (or lack of response) and the side-effect profile of the drug. Several earlier studies which reported poor outcomes of MAOIs in the treatment of depression (Medical Research Council, 1965) have not been replicated in more recent investigations (McGrath *et al.*, 1986; Murphy *et al.*, 1987; Tyrer and Shawcross, 1988). Depressed inpatients with psychotic features (experiencing hallucinations or delusions) may be refractory to PLZ (Janicak *et al.*, 1988), but these patients will often not respond to antidepressants of other classes and are difficult to treat in general. Other disorders, which overlap the depressive and anxiety disorders, such as bulimia, anorexia nervosa, social phobia and PTSD are often responsive to PLZ (Liebowitz, 1989; Gitow *et al.*, 1994; Kennedy and Goldbloom, 1994).

It is generally accepted that the clinical response will improve with the degree of MAO inhibition achieved. Patients with greater than 80% inhibition of platelet

MAO after 2 weeks of treatment do significantly better than patients with less than 80 % inhibition (Robinson *et al.*, 1973, 1978; Ravaris *et al.*, 1976; Davidson *et al.*, 1978a; Bresnahan *et al.*, 1990). The mean dose of PLZ necessary to achieve 80% MAO-B inhibition has been reported to be about 50-55 mg/day (Breshnahan *et al.*, 1990; Sharma *et al.*, 1990). Baseline MAO activity does not predict responsiveness to PLZ (Georgotas *et al.*, 1987; Breshnahan *et al.*, 1990). In order to determine if a patient will benefit from PLZ, the drug should be administered in a sufficient dose for 4-6 weeks. Following a positive response the drug should be taken for 6-12 months in order to reduce the risk of relapse (Baldessarini, 1985).

The most feared side-effect of MAOI administration is the TA-induced hypertensive crisis (Dally, 1962; Taylor, 1962; Rabkin *et al.*, 1984, 1985; Cooper, 1989). Once it is decided to initiate a trial of PLZ, the patient should be instructed to avoid TA-containing foods. TA, a substrate of MAO present in significant concentrations in certain fermented foodstuffs such as red wine, cheese, yeast extracts and pickled fish (Cooper, 1989), causes a vasopressor response, an effect that is dramatically accentuated in patients taking an MAOI. This response is in part due to the MAO-inhibited patient's inability to deaminate TA which displaces (releases) increased intracellular stores of NA (Cooper, 1989). When dietary restrictions are followed, the risk of a serious hypertensive problem is curtailed. There are also case reports of rare patients who develop idiosyncratic, spontaneous hypertensive episodes shortly after taking the initial doses of PLZ and TCP (Nies, 1984; Linet, 1986; Fallon *et al.*, 1988). Reports of minimal blood pressure rises in response to intravenous and oral TA administration in patients taking the newer reversible MAOIs suggest that these drugs will be safer and can be used without stringent dietary control (Bieck and Antonin, 1989; Cooper, 1989; Schiwy *et al.*, 1989; Stabl *et al.*, 1989; Bieck and Antonin, 1994). Although a hypertensive crisis is the most feared, a more common cardiovascular side-effect of PLZ administration is hypotension leading to dizziness and fainting after sudden postural changes (Kronig *et al.*, 1983; Cockhill and Remick, 1987; Remick *et al.*, 1989). Insomnia can be problematic, ultimately necessitating discontinuation of the drug in a small number of people (Ravaris *et al.*, 1980; Evans *et al.*, 1982; Robinson *et al.*, 1986); this side-effect can often be managed by lowering the PLZ dose (Schatzberg and Cole, 1986) or by adding a sedative drug to the therapeutic regimen at bedtime (Zimmer *et al.*, 1984; Nierenberg and Keck, 1989). Paradoxically, some patients are sedated on PLZ and take the medication prior to sleep (Tyrer, 1982). Other common side-effects include weight gain, sexual dysfunction and mania (Remick *et al.*, 1989). A discontinuation syndrome consisting of arousal, mood disturbance, and somatic symptoms has been reported with sudden cessation of PLZ administration (Tyrer, 1984; Dilsaver, 1988). It is prudent to taper the dose of PLZ over several weeks time and to avoid the administration of other antidepressants for at least 10-14 days after the PLZ is discontinued.

D.2 Biological Effects

A wide range of biologic effects is observed following acute and chronic PLZ administration. Acutely, the drug inhibits a number of enzymes including MAO-A, MAO-B, aromatic amino acid decarboxylase, tyrosine amino transferase, GABA-transaminase (GABA-T) and alanine-transaminase [ALA-T] (Popov and

Matthies, 1969; Dyck and Dewar, 1986; Baker and Martin, 1989; Yu and Boulton, 1992). Such effects may result in altered levels of NA, 5-HT, DA, GABA and ALA. Endogenous and dietary amines metabolized by MAO such as PEA, TA, octopamine and tryptamine may have important roles in the central nervous system and are elevated following PLZ administration (Juorio *et al.*, 1980; Dyck and Dewar, 1986; Baker *et al.*, 1988).

In addition to blocking the metabolism of amines, PLZ is a relatively weak releaser of DA and 5-HT from nerve terminals (Baker *et al.*, 1980; Dyck, 1984). This releasing action has been hypothesized to contribute to the acute behavioural activation seen in animal studies (Dyck, 1984). The effects of PLZ are more marked on DA than 5-HT, while NA release has not been studied to date. Several of the potential metabolites of PLZ such as PEA and TA (section D.3 of this Introduction) are also potent releasers of intracellular amines (Raiteri *et al.*, 1977). It is important to take into account the biologic activity of metabolites when considering the effects of a drug *in vivo*. Electrophysiological studies indicate that PEA may be a modulator of catecholaminergic neurotransmission with important effects on the nervous system (Paterson *et al.*, 1990). PEA also blocks the uptake and stimulates the release of DA, NA and 5-HT (Horn and Snyder, 1973; Raiteri *et al.*, 1977). PLZ itself has weak to moderate inhibitory effects on the uptake of NA, DA and 5-HT in nerve terminals (Baker *et al.*, 1978; Burgen and Iversen, 1965; Hendley and Snyder, 1968).

The original description of PLZ affecting brain GABA levels was in the German scientific literature of the 1960's and is summarized in Popov and Mathies (1969). The MAOIs PLZ, phenylpropylhydrazine and phenylvalerylhydrazine lowered GABA-T activity and elevated brain GABA following acute administration. The effects on GABA-T were blocked by pretreatment with the MAOI TCP, suggesting that interaction of PLZ with MAO, perhaps resulting in formation of an active metabolite, was important in this effect on GABA-T. Time- and dose-response studies following acute administration of PLZ have reconfirmed that PLZ causes dramatic increases in whole brain levels of GABA and alanine (ALA), another amino acid metabolized by a transaminase enzyme (Wong *et al.*, 1990b; Baker *et al.*, 1991). Following acute single-dose i.p. administration of PLZ, maximal increases in GABA and ALA whole brain levels were seen at 4 h; this increase was followed by a gradual decline over the next 24 h. The minimal dose of PLZ that produced a significant elevation in GABA and ALA levels was 5 mg/kg (0.02 mmol/kg) and 7.5 mg/kg (0.03 mmol/kg) respectively.

Stimulation of several presynaptic and postsynaptic receptors may occur subsequent to the increased synaptic levels of the amines and amino acid neurotransmitters produced by antidepressants. This has been an area of intense research in the past 2 decades. The evidence suggests that 5-HT$_2$, 5-HT$_{1A}$, α_1, α_2, and β receptors are particularly important in antidepressant mechanisms of action. Following PLZ administration, down-regulation of β-adrenoreceptors (Sellinger-Barnette *et al.*, 1980; Murphy *et al.*, 1984; Koshikawa *et al.*, 1989; McManus and Greenshaw, 1991a,b) and 5-HT$_2$ receptors (Todd *et al.*, 1992) has been reported using *ex vivo* radioligand binding and functional tests. In these studies, down-regulation was defined as a reduction in number of binding sites or a reversal of the locomotor effects of agonists for the β-adrenergic receptor. Utilizing a subtype-specific ligand, ^{125}I-iodopindolol, reduction in the density of β_1 and, to a lesser extent, β_2 adrenoreceptors was seen (Ordway *et al.*, 1991) following PLZ administration (0.02 mmol/kg/d i.p., 21d). The effects on β_2 receptors were specific to MAOIs; the tricyclic and novel antidepressants affected β_1 density only. These results require replication as PLZ and several tricyclic antidepressants affected β_1 but not β_2 receptors in another study, this time utilizing [^{3}H]-CGP 12177 as the radioligand (Paetsch and Greenshaw, 1993). The locomotor suppressant effects of the β agonist salbutamol (3 mg/kg i.p.) were attenuated following chronic administration of PLZ at a dose of 0.043 mmol/kg/d for 21 d but not at a lower dose (0.021 mmol/kg/d, 21 d) [McManus and Greenshaw, 1991b; McManus *et al.*, 1991]. In a series of studies, PLZ did not affect $GABA_B$ receptor density or affinity in rat cortex but did induce a decrease in density (B_{max}) of β-adrenoreceptors as measured by use of the radioligand [^{3}H]-dihydroalprenolol (McManus and Greenshaw, 1991a); in the same studies, PLZ had no effect on a functional measure of $GABA_B$ receptor activity (a decrease in locomotion induced by baclofen).

Using electrophysiologic experiments, PLZ administration resulted in a transient decrease in the firing rate of serotoninergic neurons followed by complete recovery (by 21 d of treatment), whereas it resulted in a persistent reduction in the firing rate of noradrenergic neurons (Blier and Montigny, 1985). This was interpreted as being due to a desensitization of the somatodendritic 5-HT autoreceptor following chronic PLZ administration (Blier and Montigny, 1985, 1994). The effects of PLZ on α_2 adrenergic, $GABA_A$ and benzodiazepine receptor function had not been studied prior to the commencement of the research projects described in this thesis.

D.3 Metabolism of PLZ

Despite years of clinical use, the metabolism of PLZ in humans is poorly understood (Robinson *et al.*, 1985; Baker and Coutts, 1989; Mallinger and Smith, 1991). Previously it was assumed that acetylation occurred, but acetylated metabolites have not been conclusively identified in man (see Section E.1 for a discussion of acetylation). At present the evidence suggests that oxidation rather than acetylation is the main metabolic pathway in humans (Figure 7). It appears that PLZ is both metabolized by and inhibits MAO, effects which are probably inseparable. Clineschmidt and Horita (1969a,b) demonstrated, *in vitro* and *ex vivo*, the presence of a metabolite of ^{14}C-labelled PLZ that co-chromatographed with phenylacetic-1-^{14}C acid (^{14}C-PAA) on thin-layer chromatography. The amount of ^{14}C-PAA recovered was diminished by pretreatment with other MAOIs. They concluded, from the results of this pioneering work, that PLZ is a substrate of MAO that is metabolized by oxidative dehydrazination to form PAA and hydrazine. Further they suggested that the hydrazine group may react irreversibly with the active site of MAO, preventing further function of the enzyme. The presence of substrates for MAO during incubation with PLZ prevents inhibition of the enzyme, supporting the idea that PLZ inhibits MAO by binding to the active site of the enzyme. A refinement of this hypothesis was presented by Tipton (1972) who suggested that the active intermediate was phenylethylidenehydrazine ($RCH_2CH=N-NH_2$). A slightly different mechanism has been proposed by Patek and Hellerman (1974), with the active intermediate being phenylethyldiazene ($RCH_2CH_2N=NH$), which is further converted to phenylacetaldehyde hydrazone. In a more recent study utilizing 1,1-dideuterated PLZ, Yu and Tipton (1989) reported results which

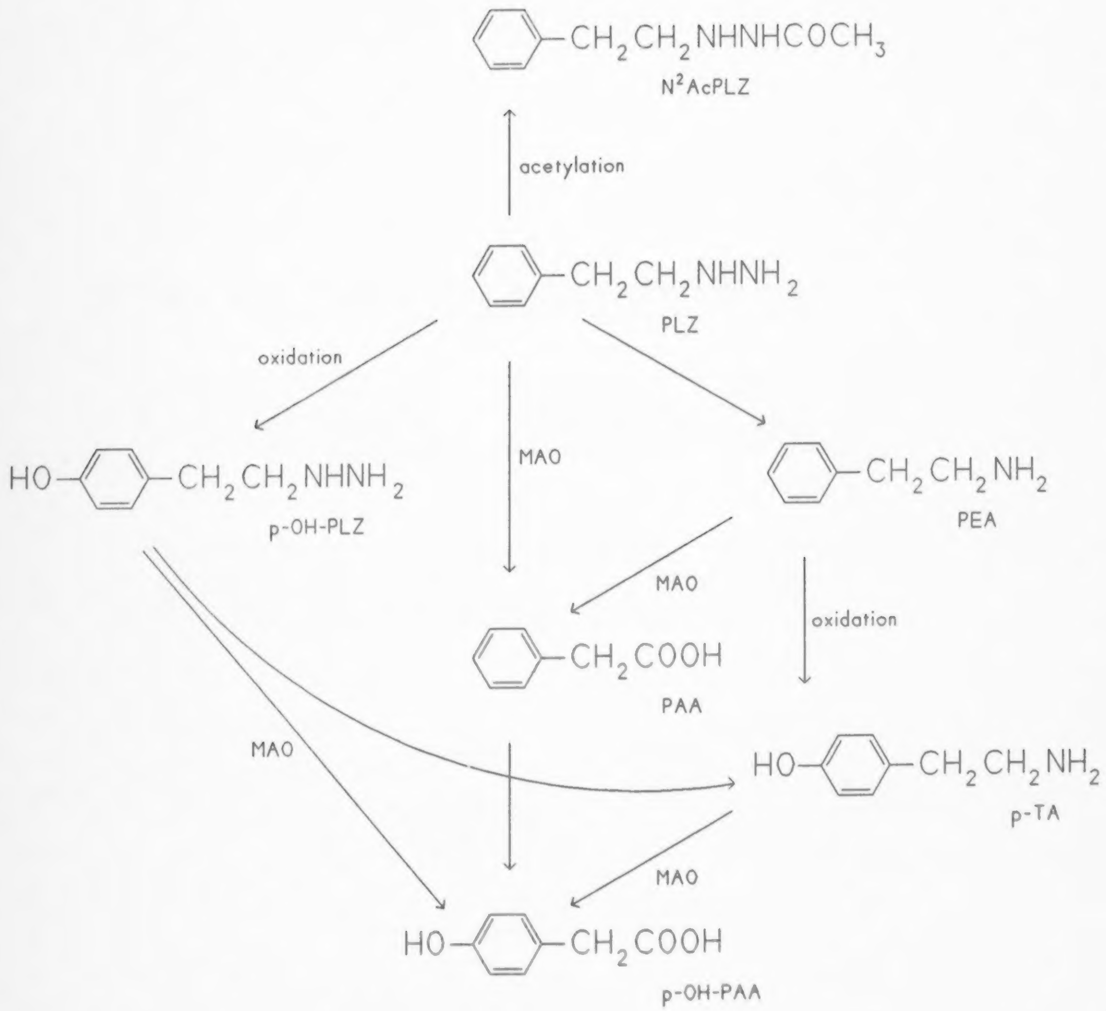
CH2CH2NHNHCOCH3
N2AcPLZ
acetylation
CH2CH2NHNH2
PLZ
oxidation
HO–CH2CH2NHNH2
p-OH-PLZ
MAO
CH2CH2NH2
PEA
MAO
oxidation
CH2COOH
PAA
MAO
HO–CH2CH2NH2
p-TA
MAO
HO–CH2COOH
p-OH-PAA

Figure 7: Possible routes of metabolism of PLZ. $RCH_2CH_2N=NH$ or $RCH_2CH=N\text{-}NH_2$ may be intermediates in going from PLZ to PAA. Hydrazine itself (H_2NNH_2) may also be an end-product of PLZ metabolism, and N-methylation of PLZ has been reported to occur *in vitro* (Yu *et al*, 1991). *p*-TA = *p*-tyramine; PAA = phenylacetic acid; *p*-OH-PAA = *p*-hydroxyphenylacetic acid; *p*-OH-PLZ = *p*-hydroxyphenelzine.

supported the notion that the irreversible inhibition of MAO by PLZ proceeds via the phenylethyldiazene intermediate, which reacts with the enzyme to form a covalent adduct. All these hypotheses postulate that PLZ is metabolized by MAO to form an active compound which is responsible for the irreversible inhibition of MAO.

In a mass spectrometric study, Robinson *et al.* (1985) found that when 6 human subjects were administered single doses of $^{13}C_6$-PLZ (30mg), 66-79% of the administered dose was excreted as either PAA or *p*-hydroxyphenylacetic acid (p-OHPAA). Labelled PLZ, or mono- or di-acetylated forms of PLZ were not detected in urine (Robinson *et al.*, 1985). In this study 21-34% of the labelled drug was not accounted for in the first 96 h after administration. As the label was incorporated on the phenyl ring rather than the side chain, the possibility of acetylhydrazine or hydrazine as a metabolite cannot be ruled out.

Another possible metabolite of PLZ is PEA, which could be deaminated to account for some of the elevation in PAA levels seen with PLZ administration. The situation is complicated by the fact that PEA is a substrate for MAO and would be expected to increase in concentration with MAO inhibition. In a study by Baker *et al.* (1982), large increases in rat whole brain concentrations of PEA were found on day 19 of PLZ administration, long after maximal MAO inhibition. Further, pretreatment with the MAOI pargyline (i.e. administration of pargyline + PLZ) resulted in larger increases in PEA levels compared to groups treated with pargyline + saline and pargyline + iproniazid. These data suggest that the elevation in PEA levels arises in part from the metabolism of PLZ to PEA, rather then being solely a consequence of MAO inhibition. This point is important as PEA is a substrate of MAO-B and inhibition of MAO would be expected to result in decreased catabolism of endogenous stores. Using a deuterated analogue of PLZ ($\alpha,\alpha,\beta,\beta$-^{2}H-PLZ) it has been confirmed in a rat model that PLZ is deaminated *in vivo* to produce PEA (Dyck *et al.*, 1985).

Yu *et al.* (1991) have reported that PLZ can be N-methylated by phenylethanolamine N-methyltransferase *in vitro*, but this route has not, to my knowledge, been investigated *ex vivo*.

The possibility of direct formation of hydroxylated metabolites of PLZ, e.g. *para*-hydroxy-PLZ (*p*-OH-PLZ) has been considered (Robinson *et al.*, 1985; Baker and Coutts, 1989), but not confirmed to date. Based on previous comparisons of PEA and its ring-hydroxylated analogue *p*-TA (Raiteri *et al.*, 1977), the presence of a hydroxyl group may increase the tendency of PLZ to affect amine (NA, DA and 5-HT) uptake and release. This possibility was addressed in a series of experiments described in this thesis.

E. N^2-ACETYLPHENELZINE

E.1 Importance as a Metabolite of PLZ

Metabolic N-acetylation is a common pathway in the metabolism of alkylamines, with acetyl coenzyme A acting as the donor and the drug as the acceptor of the acetyl group. The ability of humans to acetylate drugs in the liver is genetically determined and interindividual variation is considerable. Acetylator proficiency follows a bimodal distribution in the population, with rapid and slow acetylator groups identified (Evans *et al.*, 1960; Evans and White, 1964). In most reported studies, 50-60% of the population has the slow-acetylator phenotype. The genotype of slow acetylators is Mendelian autosomal recessive while fast acetylators may be heterozygous or homozygous dominant. A patient's acetylator phenotype may be determined by administration of an acetylatable drug (isoniazid or sulfadimidine are often used) followed by measurement of the ratio of parent to acetylated metabolite in blood or urine. Because of the similarity of PLZ to the hydrazine derivatives isoniazid and hydralazine which are known to be acetylated *in vivo* (Testa and Jenner, 1976) and because PLZ is a substrate for human N-acetyltransferases *in vitro* (Tilstone *et al.*, 1979), it has been assumed that PLZ is acetylated *in vivo* in humans. The belief that acetylation is a major pathway of PLZ metabolism has become so widespread that it is stated as a fact in several standard textbooks (Goodman and Gilman, 1980; Baldessarini, 1985).

The acetylator status of patients requiring treatment with the MAOIs was thought to predict treatment response, the severity of side-effects, and the degree of MAO-inhibition achieved, with slow acetylators showing greater clinical improvement, an increased frequency or severity of side-effects and more extensive MAO inhibition (Evans *et al.*, 1965; Johnston and Marsh, 1973; Paykel *et al.*, 1982b). However, other studies have not confirmed these observations (Davidson *et al.*, 1978b; Marshall *et al.*, 1978; Robinson *et al.*, 1978, 1980). These studies are summarized in Table 5. A number of observations and conclusions can be drawn from this research. The predictive ability of the acetylator phenotype is weak at best and has been very difficult to replicate. Knowledge of the acetylator phenotype has not led to improved patient care despite its early promise. A methodologic shortcoming in these studies is the inability to distinguish homozygous dominant from heterozygous subjects with the fast acetylator phenotype. If the hypothesis is

Table 5: Summary of studies examining the influence of acetylator phenotype on response to PLZ.

Study	*n*	*%SA*	*PLZ (mg) daily dose*	*Comparisons*	*Results*
Evans *et al.*, 1965	47	66	45 x 4 wks	clinical efficacy total side-effects severe side-effects	NSD NSD SAs>FAs
Johnstone and Marsh, 1973	72	54	90 x 3 wks	clinical efficacy	SAs>FAs
Johnstone, 1975	30	43	90 x 3 wks	clinical efficacy side-effects urinary tryptamine excretion (24 h) urinary PLZ excretion	SAs>FAs NSD SAs>FAs d 14;NSD d 21 SAs>FAs d 14
Marshall *et al.*, 1978	80	64	45-75 x 4 wks	clinical efficacy side-effects MAO activity 5-HT (blood) 5-HIAA (24 h urinary) VMA (24 h urinary)	NSD NSD NSD NSD NSD NSD
Davidson *et al.*, 1978b	16	68	up to 90 x 3 wks	clinical efficacy MAO activity	NSD NSD
Yates and Loudon, 1979	22	54	30-75 mg x 2 wks	MAO activity	NSD (d 14) SAs>FAs (d 7)
Tyrer *et al.*, 1980	60	56	45 or 90	clinical efficacy side-effects	NSD (a) NSD
Paykel *et al.*, 1982b	77	57	60-75 x 6 wks	clinical efficacy side-effects	SAs>FAs (b) NSD

SA = slow acetylator; FA = fast acetylator; d = day; h = hour; NSD = not significantly different

(a) high dose (90 mg) > low dose (45 mg)

(b) only when compared to acetylator status-matched placebo; when clinical scores of FAs and SAs directly compared, results were NSD correct, heterozygous subjects would have clinical benefits and side-effects intermediate to the homozygous genotypes. Much of this research has been based on two assumptions that are questionable. Finding a correlation between acetylator status and a clinical response (treatment efficacy or side-effects) is only indirect evidence that acetylation is an important metabolic pathway in humans: there may be other explanations for the correlation. The detection of acetylated PLZ in tissue samples would constitute direct evidence of such a pathway, and yet such attempts have been generally fruitless. Secondly, the hypothesis assumes that acetylated metabolites would be clinically and biochemically inactive, not leading to MAO inhibition, side-effects or other therapeutic effects. As has become apparent in the recent past, most psychotropic drugs, with the notable exception of lithium salts, have active metabolites. These metabolites may indeed be more active than or have opposite effects from the parent compound and have different pharmacodynamic and pharmacokinetic properties (Rudorfer and Potter, 1987; Potter and Manji, 1990; Young, 1991; Caccia and Garattini, 1992; Baker *et al.*, 1994).

At present, acetylated metabolites of PLZ have not been conclusively demonstrated in man. There has been a preliminary report of the presence of small quantities of N-acetylPLZ in the blood and urine of humans (Narasimhachari *et al.*, 1980) but the authors conclude that it is not a clinically significant metabolite. In a rat model, 1-acetyl-2-(2-phenylethyl)hydrazine (N^2-acetylphenelzine, N^2AcPLZ) [Figure 8] has been identified, using combined gas chromatography/electron impact mass spectrometry, as a metabolite of PLZ (Mozayani *et al.*, 1988). At one hour post-injection of PLZ (0.21 mmol kg^{-1} i.p.) small quantities of N^2AcPLZ were identified in the blood (33.6 - 41.0 ng/g) and brain (84.5 - 132.0 ng/g). This work

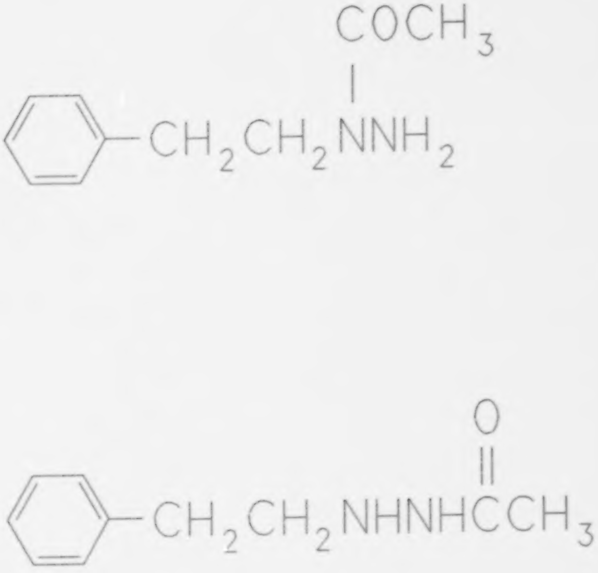

Figure 8: Structure of N¹AcPLZ (top) and N²AcPLZ (bottom).

has since been replicated and extended, indicating that N^2AcPLZ is present in rat blood and liver, albeit in extremely low quantities following PLZ (0.38 mmol kg^{-1} i.p.) administration (Coutts *et al.*, 1991) [Table 6]. In these studies, no evidence of the other possible acetylated analogues of PLZ, i.e. 1-acetyl-1-(2-phenylethyl)hydrazine (N^1AcPLZ) or 1,2-diacetyl-1-(2-phenylethyl)-hydrazine (N^1N^2-diAc-PLZ), was found. It is thus confirmed in rat that acetylation is a minor metabolic pathway of PLZ.

E.2 Neurochemical Properties

The assumption that acetylated metabolites of PLZ would be inert was disproven when studies on N^2AcPLZ indicated that it is an MAOI that merits further study as a drug in its own right (Danielson *et al.*, 1984, 1988; Coutts *et al.*, 1991). Ninety min following administration of N^2AcPLZ (0.1 and 0.2 mmol kg^{-1} i.p.) greater then 80% inhibition of MAO-A and -B has been reported, resulting in significant elevations of brain levels of 5-HT (both doses), NA and DA (at a dose of 0.2 mmol kg^{-1}) [Coutts *et al.*, 1991]. It was concluded in this study that the tissue levels of N^2AcPLZ were probably not sufficient to contribute significantly to the elevation in brain amines seen during PLZ administration. Metabolic conversion of N^2AcPLZ to PLZ did not occur to any significant degree. N^2AcPLZ was slightly weaker than PLZ at producing inhibition of MAO-A and -B, and the authors suggested that this reduced efficacy of N^2AcPLZ relative to PLZ as an MAO inhibitor *in vivo* might be due to reduced potency and/or restricted entry into the brain.

Table 6: Levels of PLZ and N^2AcPLZ in rat blood and liver after intraperitoneal administration of PLZ (dose of 0.38 mmol/kg). Data reported as mean ± SEM; n = 5 (Coutts *et al.*, 1991).

	Blood Level, ng/g		Liver Level, ng/g	
Time (min)	PLZ	N^2AcPLZ	PLZ	N^2AcPLZ
15	187 ± 24	14 ± 2	222 ± 5	7 ± 1
30	765 ± 12	25 ± 2	327 ± 17	49 ± 5
45	1144 ± 37	59 ± 3	958 ± 91	103 ± 8
60	1724 ± 15	36 ± 4	336 ± 42	82 ± 6
120	108 ± 15	20 ± 2	153 ± 9	36 ± 1
180	24 ± 5	10 ± 1	46 ± 7	28 ± 4

F. γ-AMINOBUTYRIC ACID (GABA)

F.1 Biochemistry, Metabolism and Receptors

GABA, a major inhibitory neurotransmitter in the mammalian central nervous system (Roberts *et al.*, 1976), is distributed widely through the brain and spinal cord, being used by as many as 40% of all neurons (Guidotti *et al.*, 1983) but absent in the periphery except for minute quantities (Erdö and Wolff, 1990). Most GABA-containing neurons are interneurones, but a small number of tracts, including the strio-nigral, nigro-thalamic and nigro-tectal, are observed. The concentration of GABA in the brain is 2-3 orders of magnitude larger than that of the amine neurotransmitters (Cooper *et al.*, 1991).

The metabolism of GABA is associated intimately with carbohydrate metabolism (Figure 9). One of the precursors (α-ketoglutaric acid) and the product (succinic acid) of GABA metabolism are components of the Kreb's cycle. Glutamic acid decarboxylase (GAD) catalyzes the α-decarboxylation of L-glutamate to form GABA. GAD has a regional distribution that parallels that of GABA, resulting in it being a valuable marker of GABA neurons. GAD is a cytosolic enzyme that requires pyridoxal phosphate as a co-factor and is inhibited by agents that bind to the co-factor. GABA-transaminase (GABA-T), also dependent on pyridoxal phosphate, catalyzes the conversion of GABA to succinic semialdehyde and of α-ketoglutaric acid to L-glutamate. Succinic semialdehyde is then converted to succinic acid and utilized in the Kreb's cycle (DeLorey and Olsen, 1994). Endogenous levels of GABA rise rapidly postmortem due to unopposed GAD activity; GABA-T is inactivated under anaerobic conditions (Cooper *et al.*, 1991).

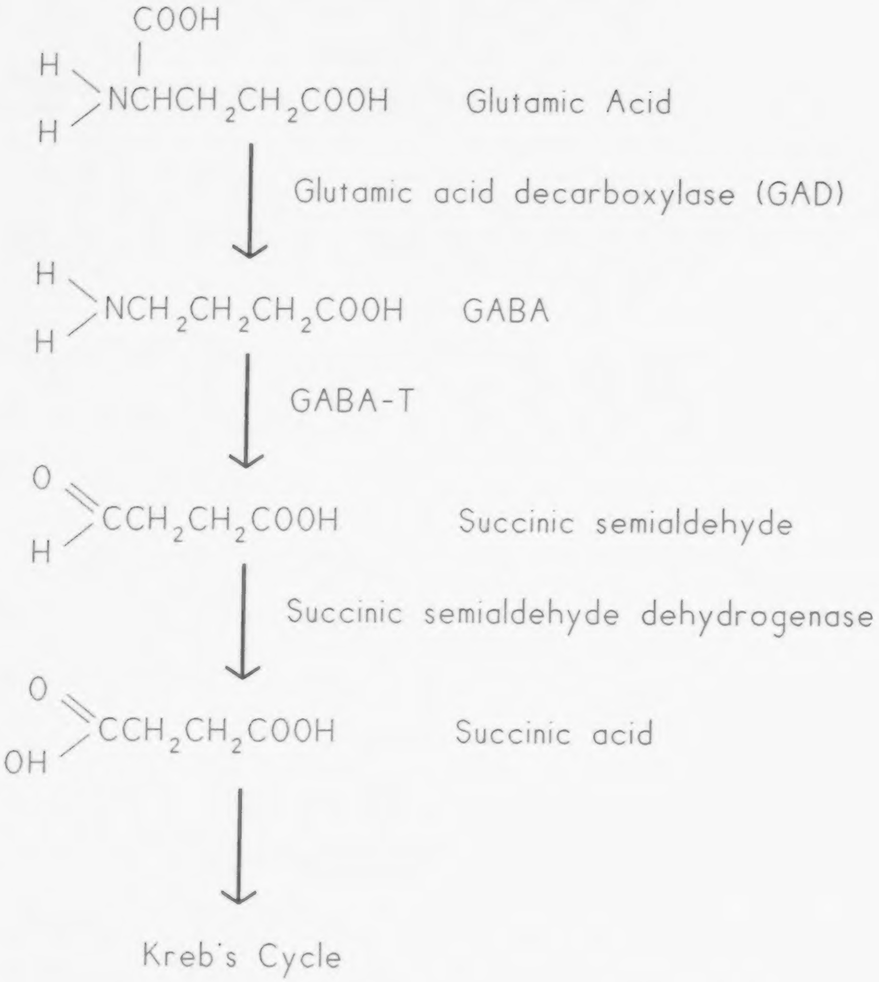
COOH
H
H
NCHCH2CH2COOH
Glutamic Acid
Glutamic acid decarboxylase (GAD)
H
H
NCH2CH2CH2COOH
GABA
GABA-T
O
H
CCH2CH2COOH
Succinic semialdehyde
Succinic semialdehyde dehydrogenase
O
OH
CCH2CH2COOH
Succinic acid
Kreb's Cycle

Figure 9: The formation and breakdown of GABA.

Two major forms of GABA receptor have been described; $GABA_A$ and $GABA_B$ (Bowery *et al.*, 1984). $GABA_A$ receptors are bicuculline (antagonist)- and muscimol (agonist)-sensitive and baclofen-insensitive. They are complex heteropolymeric ionotropic receptors, composed of five subunits surrounding a chloride ion channel (Bristow and Martin, 1990; Nayeem *et al.*, 1994). The $GABA_A$ receptor shares amino acid sequence and structural similarities with other ionotropic receptors including nicotinic acetylcholine and glycine receptors, leading to the idea of a superfamily of ligand-gated receptors. Stimulation of the receptor by GABA results in a flux of chloride ions that is dependent on local concentration and electrical gradients; this usually hyperpolarizes and stabilizes the membrane potential. Agents that inhibit $GABA_A$ receptor function tend to induce convulsions.

The $GABA_A$ receptor contains binding sites for benzodiazepines and barbituates (Fischer and Olsen, 1986). Benzodiazepines act upon specific subunits of the $GABA_A$ supramolecular complex to influence the affinity of the GABA receptor for GABA, an effect known as allosteric modulation (Costa *et al.*, 1975; Haefely *et al.*, 1975; Haefely, 1989a,b; Richards *et al.*, 1991). The benzodiazepine receptor exhibits complex pharmacology, being affected by agonists, antagonists and inverse agonists (Bruun-Meyer, 1987; Costa,1989). Agonists facilitate GABAergic function, antagonists block the effect of agonists but otherwise have no direct effect and inverse agonists inhibit GABAergic responsiveness (Zorumski and Isenberg, 1991). The benzodiazepine receptor is the only receptor hypothesized to have an inverse agonist to date, but other examples likely exist. An enhanced affinity of the benzodiazepine receptor for benzodiazepines in the presence of GABA has also been described (Martin and Candy, 1978).

Molecular genetic and immunocytochemical studies have revealed a plethora of isoforms of the subunits of the $GABA_A$ receptor (Wisden *et al.*, 1988; Pritchett *et al.*, 1989; Luddens and Wisden, 1990; Fritschy *et al.*, 1992) but the precise subunit composition of any $GABA_A$ receptor *in vivo* is presently unknown. Transient expression studies have demonstrated the importance of the subunit isoform composition to the recognition properties and functional characteristics of the resulting oligomer. Receptors containing the α_1 subunit displayed the Bz1 phenotype (high affinity for CL218872 and the β-carbolines) while those containing the α_2 or the α_3 subunit exhibited the Bz2 phenotype. Recombinant receptors containing the α_5 subunit appear to exhibit the Bz2 phenotype but show a very low affinity for the imidazopyridine, zolpidem (Pritchett and Seeburg, 1990). Those containing the α_4 or α_6 subunit display a very low affinity for the classical benzodiazepine receptor agonists but a high affinity for the partial inverse agonist Ro154513 (Sieghart *et al.*, 1987; Malminiemi and Korpi, 1989; Luddens *et al.*, 1990; Wisden *et al.*, 1991).

The $GABA_B$ receptor is a metabotropic receptor which affects activity of adenylate cyclase and the function of Ca^{2+} and K^+ ion channels. It is likely that several subtypes of $GABA_B$ receptor, each with a different transduction pathway, exist (Scherer *et al.*, 1988). The $GABA_B$ receptor was not studied in this thesis since a series of experiments examining the effects of PLZ and other antidepressants on this receptor has recently been completed in our laboratory (McManus and Greenshaw, 1991a,b).

F.2 GABA and Psychiatric Disorders

Several neurologic and psychiatric disorders including Huntington's chorea (Perry *et al.*, 1973), Parkinson's disease (Lloyd and Hornykiewicz, 1975), epilepsy (Morselli and Lloyd, 1983) and tardive dyskinesia (Fibiger and Lloyd, 1984) have been related to disturbed GABA function (Barchas *et al.*, 1994). In psychiatry, GABA has been strongly associated with the anxiety disorders but recent findings also suggest a role in the affective disorders. Decreased GABA levels in cerebrospinal fluid (Gold *et al.*, 1980; Kasa *et al.*, 1982; Gerner and Hare, 1981; Gerner *et al.*, 1984; Gerner and Fairbanks, 1986; Berrettini *et al.*, 1984) and plasma (Petty and Schlesser, 1981; Petty and Sherman, 1982; Petty *et al.*, 1990) have been reported in depressed patients. Patients suffering from bipolar affective disorder have also been reported to have lower CSF (Kasa *et al.*, 1982) and plasma (Berrettini *et al.*, 1982; Petty *et al.*, 1990, 1993a,b) levels of GABA . The decrease in plasma or CSF GABA levels has been hypothesized to represent a trait (vs state) marker of mood disorders (Petty *et al.*, 1990, 1993a,b). A number of other studies, however, have found more inconsistent results. Postmortem studies of depressed patients have not found changes in GABA levels (Korpi *et al.*, 1988) or forebrain $GABA_B$ binding sites (Cross *et al.*, 1988) relative to values in normal controls. GAD activity has been found to be decreased in the frontal cortex and striatum in one study (Perry *et al.*, 1977) but not in another (Cheetham *et al.*, 1988). Both GAD and GABA-T activity were found to be decreased in the blood of depressed patients compared to controls (Berrettini *et al.*, 1980; Kaiya *et al.*, 1982).

In general, the literature examining the role of GABA in the etiology of anxiety is sparse other than documenting the efficacy of benzodiazepines in the alleviation of symptomatology. Although an essential area of investigation, plasma and CSF levels of GABA have not been studied to date in patients with anxiety disorders. A recent line of research has examined functional responses of the benzodiazepine receptor in patients with PD. Altered sensitivity was found when patients with PD were exposed to an agonist (diazepam) or antagonist (flumazenil) of the benzodiazepine receptor (Nutt *et al.*, 1990; Roy-Byrne *et al.*, 1990). Marazziti *et al.* (1994) reported that the density of peripheral benzodiazepine receptors on platelets was significantly lower in PD patients than in controls, but Rocca *et al.* (1990) had earlier reported that the density of such receptors in lymphocytes was lower than control values in patients with generalized anxiety disorder but not in those with PD. There is now a relatively large body of evidence indicating that the neuropeptide cholecystokinin is a panicogenic agent (Bradwejn and Koszycki, 1994). In this regard, it is interesting that cholecystokinin and GABA are colocalized in neurons in cortex, hippocampus and amygdala (Kosaka *et al.*, 1985), that release of cholecystokinin from rat cortex is reduced by GABA or diazepam (Yaksh *et al.*, 1987) and that cholecystokinin increases K^+-evoked release of GABA from brain slices (Pérez de la Mora *et al.*, 1993).

A number of drugs which directly affect GABAergic function have therapeutic potential in psychiatry. Many of the drugs used in the stabilization of bipolar patients such as carbamazepine, valproic acid and clonazepam enhance GABAergic function. The GABA agonist progabide has been reported equally efficacious to IMI in the treatment of patients with depression (Morselli *et al.*, 1981). In behavioural studies in laboratory animals, GABA agonists such as fengabine, baclofen and muscimol have been reported to have effects similar to antidepressants (Delina-Stula and Vassout, 1978; Lloyd *et al.*, 1983, 1987a,b, 1989; Singh *et al.*, 1986). There is considerable controversy, however, as Paykel *et al.* (1991) have not found GABA agonists to be efficacious as antidepressants clinically, and they are not used clinically at present. Baclofen, a $GABA_B$ agonist, has been reported useful in the treatment of PD (Breslow *et al.*, 1989). The benzodiazepines are used widely in the treatment of epilepsy, drug withdrawal and the anxiety disorders. Alprazolam, a triazolobenzodiazepine, has been reported in methodologically sound studies to possess antidepressant activity (Rickels *et al.*, 1985, 1987; Eriksson *et al.*, 1987; Mooney *et al.*, 1988; Warner *et al.*, 1988; Kravitz *et al.*, 1990; Rimon *et al.*, 1991) in addition to its beneficial effects in PD and generalized anxiety disorder (Ballenger, 1993; Bradwejn, 1993).

Antidepressants of the more traditional types have also been found to affect GABA. The TCA DMI and, to a lesser extent, IMI and trimipramine (TMI) were found to increase GABA release from the rat thalamus after acute administration (Korf and Venema, 1983). Similarly, IMI has been found to increase endogenous GABA release following 4 days of continuous administration (Sherman and Petty, 1982). Of particular importance to the studies described in this thesis has been the description of the ability of MAOIs such as PLZ (Popov and Matthies, 1969; Perry and Hansen, 1973; Baker *et al.*, 1991), iproniazid (Balzer *et al.*, 1960) and pargyline (Schatz and Lal, 1971; Patel *et al.*, 1975) to elevate brain levels of GABA, presumably by inhibition of the catabolic enzyme GABA-T. Administration of hydrazine has also been found to cause GABA-T inhibition and elevated GABA levels in rat brain (Perry *et al.*, 1981).

Chronic administration of antidepressants and benzodiazepines may affect GABA and benzodiazepine receptor density. A wide range of antidepressant treatments including ECT, lithium salts, and carbamazepine have been associated with increased $GABA_B$ density following chronic treatment (Lloyd *et al.*, 1985, 1989; Gray and Green, 1987; Motohashi *et al.*, 1989). This up-regulation has been disputed, however, in a number of studies (Cross and Horton, 1987; Szekely *et al.*, 1987; McManus and Greenshaw, 1991a,b). Several antidepressant drugs, including DMI, bupropion, maprotiline, zimelidine (5-HT uptake inhibitor) and adinazolam (a triazolobenzodiazepine) have been reported to decrease the number of benzodiazepine receptors in rat cortex using $[^3H]$-flunitrazepam as the radioligand (Suranyi-Cadotte *et al.*, 1984, 1990; Barbaccia *et al.*, 1986). However, Kimber *et al.* (1987) were unable to find an effect of chronic administration of DMI and TCP on $[^3H]$-flunitrazepam binding. Przegalinski *et al.* (1987) in a study in mice found no change in ^3H-flunitrazepam binding after administration of IMI, DMI or AMI for 21 days. Using a microiontophoretic technique, Bouthillier and de Montigny (1987) found that 3 week treatment with DMI, TMI and citalopram reduced the effect of flurazepam application on cholycystokinin-induced activation of hippocampal pyramidal neurons, suggesting that chronic antidepressant treatment is associated with down-regulation of benzodiazepine receptors. These findings have not been extensively studied and require further work to ascertain their generalizability. At the time of commencement of the research project described in this thesis, the effects of PLZ on $GABA_A$ and benzodiazepine receptors had not been studied. Since PLZ dramatically affects GABA levels, the effect on $GABA_A$ and benzodiazepine receptor density and function following chronic treatment warrants further study.

G. OBJECTIVES

The objectives of the research described in this thesis were to examine:

(1) The neurochemical effects of acute and long-term administration of PLZ compared with N^2AcPLZ in rodents. Although N^2AcPLZ appears to be a very minor metabolite of PLZ, its rather strong inhibitory effects on MAO suggest that it may be a potentially useful antidepressant in its own right and worthy of further investigation. Of particular interest were the effects of both drugs on brain levels of neurotransmitters (NA, DA, 5-HT, GABA and glycine), aliphatic amino acids (ALA, valine, isoleucine, leucine), metabolites of the classical neurotransmitter amines (DOPAC, HVA and 5-HIAA), enzyme activity (MAO-A, MAO-B, GAD, GABA-T) and neurotransmitter receptor function, density and affinity (α_2-adrenergic, $GABA_A$ and benzodiazepine receptors).

(2) The possibility of ring hydroxylation as a metabolic pathway of PLZ metabolism in rodents and human subjects. The effects of *p*-OH-PLZ on amine (NA, DA, 5-HT) uptake and release compared to those of PLZ were also to be investigated.

(3) The effects of PLZ administration on urinary excretion of: 5-HT; metabolites of classical neurotransmitter amines; and trace amines (PEA, TA, tryptamine [T]) in psychiatric patients. Although there have been scattered reports on the effects of PLZ on urinary excretion of some amines or metabolites (Dewhurst, 1968; Murphy *et al.*, 1977; Bieck *et al.*, 1984; Antonin *et al.*, 1985; Baker *et al.*, 1985), no comprehensive study on its effects on several of the types of the amines thought to be important in the etiology of depression had been conducted.

MATERIALS AND METHODS

A. CHEMICALS

Table 7: Chemicals used in the studies described in this thesis.

Chemicals	**Suppliers**
acetic acid, glacial	BDH Chemicals (Toronto, ON)
acetic anhydride	Caledon Laboratories (Georgetown, ON)
acetonitrile, HPLC grade, DIG	BDH
L-[2,3-^{3}H]-alanine	Amersham Canada Ltd. (Oakville, ON)
L-alanine	Aldrich Chemical Co. (Milwaukee, WI)
γ-aminobutyric acid	Aldrich
aminobutyric acid, γ-[2,3-^{3}H(N)]	Dupont Canada (Mississauga, ON)
2-aminoethylisothiouronium bromide	Sigma Chemical Co. (St. Louis, MO)
ammonium hydroxide	Fisher Scientific (Mississauga, ON)
ascorbic acid	Fisher
benzylamine	Sigma
bovine serum albumin	Sigma
calcium chloride	Fisher
chloroform, reagent grade	Fisher
p-chlorophenylacetic acid	Aldrich
2-(4-chlorophenyl)ethylamine	Sigma
citric acid	Anachemia Ltd. (Edmonton, AB)
clonazepam	Research Biochemicals Inc. (Natick, MA)
clonidine	Sigma
cupric sulfate	Fisher
cyclohexane	Fisher

dicyclohexylcarbodiimide	Aldrich
diethyl ether	BDH
di-(2-ethylhexyl)-phosphate	Sigma
3,4-dihydroxyphenylacetic acid	Sigma
dithiothrietol	Sigma
dopamine, 3,4-[7-^{3}H]-	Dupont
dopamine HCl	Sigma
ethyl acetate	BDH
ethylenediamine tetraacetate, disodium salt	Fisher
flunitrazepam, [methyl-^{3}H]-	Dupont
Folin phenol reagent	Sigma
glucose	Fisher
glusalase	Calbiochem (La Jolla, CA)
glutamate, sodium	Sigma
glutamic acid, L-[1-^{14}C]-	Amersham
glutathione	Sigma
glycerol	Sigma
L-glycine	Sigma
hexafluoroisopropanol	Pierce Chemicals (Rockford, IL)
homovanillic acid	Sigma
hydrochloric acid, 37-38%	Fisher
p-hydroxybenzyl alcohol	Sigma
5-hydroxyindole-3-acetic acid	Sigma
p-hydroxyphenelzine	synthesized by Dr. B. Davis, University of Saskatchewan (Saskatoon, SA)
m-hydroxyphenylacetic acid	Sigma

p-hydroxyphenylacetic acid	Sigma
p-hydroxyphenylpropionic acid	Sigma
hydroxytryptamine binoxalate, 5-[2-^{14}C]	Dupont
5-hydroxytryptamine creatinine sulfate	Sigma
5-hydroxy-[G-^{3}H] tryptamine creatinine sulfate	Amersham
iprindole HCl	Wyeth Research (Taplow, U.K.)
D,L-isoleucine	Sigma
iso-pentane	BDH
isobutyl chloroformate	Aldrich
α-ketoglutarate	Sigma
L-leucine	Sigma
magnesium sulfate	Fisher
3-methoxy, 4-hydroxyphenyl-ethylene glycol	Calbiochem
3-methoxytyramine HCl	Sigma
5-methyltryptamine HCl	Aldrich
muscimol	Sigma
muscimol, [methylene-^{3}H(N)]	Dupont
nialamide	Sigma
nicotinamide adenosine dinucleotide	Sigma
(-)-noradrenaline HCl	Sigma
noradrenaline, levo-[7-^{3}H]-	Dupont
D,L-norleucine	Aldrich
normetanephrine HCl	Sigma

octyl sodium sulfate	Eastman Kodak Co. (Rochester, N.Y.)
pargyline HCl	Sigma
pentafluorobenzenesulfonyl chloride	Aldrich
pentafluorobenzoyl chloride	Aldrich
pentafluorophenol	Aldrich
pentafluoropropionic anhydride	Aldrich
perchloric acid, 60%	Fisher
phenelzine sulfate	Sigma
β-phenylethylamine HCl	Sigma
phenyl-3-propylamine	Sigma
phenylacetic acid	Sigma
phenylethylamine HCl, 2[ethyl-1-^{14}C]	Dupont
phosphoric acid, 85%	Fisher
potassium bicarbonate	Fisher
potassium biphosphate	J.T. Baker Chemical Co. (Phillipsberg, N.J.)
potassium carbonate anhydrous	Fisher
potassium chloride	Fisher
potassium dihydrogen orthophosphate	Fisher
Protosol	Dupont
pyridoxal phosphate	Sigam
saline, 9%	Fisher
scintillation fluid (Ready Safe)	Beckman Instruments Inc. (Mississauga, ON)
sodium acetate	Sigma

sodium bicarbonate	Fisher
sodium borate	Fisher
sodium carbonate anhydrous	Fisher
sodium chloride	Fisher
sodium deoxycholate	Fisher
sodium hydroxide	Fisher
sodium phosphate, dibasic, anhydrous	Fisher
sodium phosphate, monobasic	Fisher
sodium potassium tartrate	Allen & Hanbury's (Toronto, ON)
sucrose	Fisher
sulfuric acid	Fisher
toluene, DIG	BDH
toluene, reagent grade	BDH
(±)-tranylcypromine HCl	Sigma
tri-*n*-octylamine	Sigma
trifluoroacetic anhydride	Aldrich
TRIS (tri[hydroxymethyl]aminomethane)	Fisher
Triton X-100	Terochem Laboratories Ltd. (Edmonton, AB)
tryptamine HCl	Sigma
tryptophan	Sigma
m-tyramine HCl	Vega Chemicals (Tuscon, AZ)
p-tyramine HCl	Sigma
D,L-valine	Nutritional Biochemical Corp. (Cleveland, OH)

B. ANALYTICAL INSTRUMENTATION AND APPARATUS

B.1 Gas Chromatography (GC)

Levels of amino acids (GABA, ALA, glycine, leucine, isoleucine, and valine), trace amines (PEA, TA, T), neurotransmitter metabolites (MHPG, HVA, 5-HIAA, 3-MT, NME), drug (PLZ), and potential drug metabolites (*p*-OH-PLZ, PAA, m-OH-PAA, p-OH-PAA) were measured using a gas chromatograph following derivatization with suitable reagents (details are given in later sections). GC was performed on a Hewlett Packard (HP) 5890 gas chromatograph (Hewlett Packard, Palo Alto, CA, U.S.A.) equipped with a fused silica capillary column [cross-linked 5% phenylmethylsilicone phase, 0.31 mm I.D. x 25 m (Hewlett Packard)], an electron-capture detector with a radioactive source of 15 mCi Nickel-63, an HP 7673A automatic sampler and an HP 3392A integrator for recording/integrating chromatographic peaks. Helium, the carrier gas, was set at a flow rate of 2 ml/min. Argon-methane (95%-5%) at a flow rate of 35 ml/min was used as makeup gas to the detector. The injection port temperature was 200°C and the detector temperature was 325°C.

B.2 High Pressure Liquid Chromatography with Electrochemical Detection (HPLC-EC)

Brain levels of NA, DA, 5-HT, tryptophan (TRP), HVA, DOPAC and 5-HIAA were measured using reverse-phase HPLC. This methodology consisted of a solvent delivery system set at a flow rate of 1 ml/min (Model 510, Waters Associates, Milford, MA, USA) coupled to an automatic injector (WISP, Waters model 710B) which was set to inject 15 μl samples. Compounds of interest were separated on an Econosphere-C_{18} column (4.6 mm x 250 mm, 5 μm particle size, Applied Science Labs, Avondale, PA, USA). A precolumn (4.6 mm x 30 mm) with the same packing material as that in the analytical column was used routinely. Eluants from the column were detected by an electrochemical detector (model 460, Waters) with the applied potential set at 0.85 v. Chromatographic peaks were recorded and integrated using a model 750 integrator (Waters). The mobile phase for the HPLC system consisted of $NaH_2PO_4.H_2O$ (55 mM), octyl sodium sulfate (0.85 mM), Na_2EDTA (0.37 mM) and acetonitrile (9% v/v). The solution was filtered through a type HA filter (0.45 μm, Millipore) before being degassed and adjusted to a pH value of 3.0 with phosphoric acid.

B.3 Ultraviolet Spectrophotometer

A Pye Unicam SP 1700 (Pye-Unicam, Cambridge, UK) visible/ultraviolet spectrophotometer was used for determination of protein concentrations in receptor binding homogenates. A deuterium-tungsten lamp was used as a light source and the wavelength was set at 660 nm.

B.4 Liquid Scintillation Spectrometry

A Beckman LS 7500 (Beckman Co., Fullerton, CA, USA) liquid scintillation spectrometer coupled to a Datamex 43 printer was used for counting radioactivity in all radioligand-receptor binding, radioenzymatic and uptake and release assays. The liquid scintillation cocktail used was Beckman Ready Safe®.

B.5 Locomotor Activity Monitoring System

The activity monitoring system (Acadia Insts. Ltd., Saskatoon, Saskatchewan, Canada) consisted of six 17" x 17" x 12" plexiglass test cages each placed in a 12 x 12 beam infra-red grid system (Acadia Infra-red Grid Model 17-12 with vertical sensors). The test cage sensors were interfaced with a microcomputer system (Acadia 6502 Data Gatherer) for data-logging and temporal analysis of activity counts.

B.6 Superfusion Release Apparatus

The apparatus for the study of the release of neurotransmitters (NA, 5-HT and DA) from nerve terminals was a Brandel SF-600 Superfusion system (Biomedical Research and Development Laboratories Inc., Gaithersburg, MD). This apparatus permits superfusion of six tissue chambers simultaneously.

B.7 Uptake Apparatus

The apparatus for the study of the inhibition of uptake of neurotransmitters (NA, DA and 5-HT) consisted of a glass filtration flask (trap) attached to a vacuum line and a filtration system obtained from the Millipore Corporation (Mississauga, ON). The Millipore filtration system consisted of a 20 cm diameter tank, a cover with 12 spaces for filter papers, and a head with a screw-on compression nut.

B.8 Tissue Homogenizer

Tissue samples (brain, heart, and liver) were homogenized using a variable speed Tri-R homogenizer (Model S63C, Tri-R Instruments, Rockville, NY, USA) with a Teflon® glass pestle and a glass grinding tube. The rotor shaft had a maximum speed of 12,000 rpm with a ten-speed setting (setting of 7 was used routinely).

B.9 Tissue Chopper

A McIllwain tissue chopper (Brinkman Instruments, Rexdale, ON) was used to make prisms (0.1 x 0.1 x approx. 2 mm) from hypothalamic and striatal tissue for release and uptake experiments.

B.10 Centrifuges

A Sorvall GLC-2B or Sorvall GLC-1 General Laboratory Centrifuge (Dupont Instruments) was used for low-speed, small volume centrifugations. Higher speed and/or larger volume centrifugations were carried out in a Damon-IEC B-20A (Damon/IEC, Needham Hts., MA, USA) refrigerated high-speed centrifuge or a Beckman L755 refrigerated ultracentrifuge.

B.11 Filtration

A Brandel Cell Harvester (M24R, Brandel, Gaithersburg, MD, USA) equipped with Whatman GF/C filters was used for the filtration step in [^{3}H]-muscimol and [^{3}H]-flunitrazepam receptor binding assays.

B.12 Shaker-Mixer

Two types of vortex-shakers were used: Ika-Vibrax VXR2 Shaker (Janke and Kunkel Instruments, Staufer, Germany) and a Thermolyne Maxi Mix vortex mixer (Sybron/Thermolyne Instruments, Dubuque, IA, USA).

B.13 Weighing Balances

A Mettler AE 160 electronic balance was used for weighing chemicals and biological samples. Animals were weighed on a 700 series triple beam balance with a 2610 g capacity (Ohaus, Florham Park, NJ, USA).

B.14 pH Meter

The pH values of the various buffer solutions were determined with either an Accumet model 610 or model 915 pH meter (Fisher Scientific, Fairlawn, NJ, USA) standardized with a certified buffer solution (pH 7.00 ± 0.01; Fischer Scientific).

B.15 Glass Cleaning

All glassware was rinsed with tap water and washed with biodegradable Sparkleen solution (Fisher Scientific Co., Fairlawn, NJ, USA). Further washing was accomplished with a dishwasher (Miele Electronic 6715, Miele Electronics, FRG). For test tubes, an additional cleaning step was added; test tubes were sonicated (Ultra-sonic cleaner, Mettler Electronics) in a solution of Decon 75 concentrate (BDH Chemicals) before the dishwasher wash. All glassware was then air-dried in a mechanical convection oven (Model 28, Precision Scientific Group, Baxter Corp., Edmonton, AB) at 250°C for 1 h. In the case of assays utilizing radiochemicals, the glassware was soaked for a minimum of 2 days in a solution of Conrad-70 (Baxter/Canlab, Mississauga, ON), rinsed, sonicated and cleaned as above.

C. ANIMALS

Male Sprague Dawley rats weighing 200-300 g were purchased from Bioscience Animal Services, Ellerslie, Alberta. The animals were group housed (2 per cage) under a 12 h light/dark cycle at a temperature of 20 ± 1°C. Food and water were freely available. The animal feed (Lab-Blox Feed, Wayne Feed Division, Continental Grain Co., Chicago, USA) composition was 4.0% crude fat (min), 4.5% crude fibre (max) and 24% crude protein (min).

C.1 Surgery and Administration of Drugs

All doses of drugs administered to animals are expressed as mmol/kg of free base. During acute studies, drugs were dissolved in a suitable vehicle and administered intraperitoneally [with a 1 ml tuberculin syringe equipped with a 27G½ needle (Becton Dickson, Closter, NJ, USA)] at a concentration such that animals received 2 ml/kg. In the chronic study, each animal was deeply anesthetized with a mixture of ether and air, and an osmotic minipump (Alzet 2ML4, Alza Corp., Palo Alto, CA) was implanted subcutaneously in the dorsal thoracic region. Each pump was filled with a drug solution individually adjusted in concentration (Greenshaw, 1986) or with the distilled water vehicle according to each animal's group allocation to provide constant infusion for a total of 28 days. The incisions were sutured and, after recovery, the animals were placed in normal housing conditions. These procedures had been approved by the Health Sciences Animal Welfare Committee, University of Alberta.

C.2 Sample Collection and Storage

At a predetermined time period after drug injection, animals were killed by instant guillotine decapitation; tissues including whole brain (meninges and pineal gland removed), liver and heart were dissected out and frozen immediately in isopentane over solid carbon dioxide. Brain tissue was stored at -80°C, while heart and liver samples were stored at -20°C, until the time of analysis. Animals were sacrificed in the early afternoon (1200-1500 h) to control for diurnal variations.

D. HUMAN SUBJECTS

D.1 Administration of Phenelzine

Following explanation of the study and receipt of informed consent, human subjects with psychiatric disorders were treated with PLZ sulfate (15 mg tablets). Subjects were started at a 15 mg BID dosage that was increased slowly to therapeutic effect, or until intolerance of side effects was realized. All subjects were instructed to follow a low amine diet (Cole and Schatzberg, 1983) 48 h prior to and during treatment with PLZ. Subjects were not taking other psychotropic mediations, including antidepressants or sedatives.

D.2 Sample Collection and Storage

Prior to starting PLZ, and following 2 and 4 weeks of treatment, a 24-h urine sample was obtained from each patient. The 24-h urine sample containing EDTA (20 ml, 2%) was divided into aliquots and stored at -20°C following measurement of the total volume.

D.3 Clinical Measures

Diagnoses based on DSM-III-R were made following review of the patient's history. A Clinical Global Impression of Improvement Scale (CGI-I) was completed at 4 weeks of treatment. All procedures involving human subjects were submitted for prior approval by the Research Ethics Board, Faculty of Medicine, University of Alberta.

E. ANALYTICAL METHODS

E.1 Determination of MAO Activity

Activity of MAO in brain, liver and heart was determined using a modification of the radiochemical procedure of Wurtman and Axelrod (1963) with 5-HT and PEA as substrates for MAO-A and -B respectively. The tissues were homogenized in 5 vol of ice-cold isotonic KCl and then further diluted to 20 vol with isotonic KCl. Of this dilute homogenate, 25 μl were added to each tube (for blank controls 25 μl of isotonic KCl). All tubes were placed on ice and sodium phosphate buffer (250 μl, O.5 M, pH=7.4) was added to each. Aliquots (25 μl) of [^{14}C]-5-HT and [^{14}C]-PEA, suitably diluted with unlabelled compound, were added to each tube to give final substrate concentrations of 100 and 25 μM, respectively. The tubes were incubated at 37°C for 20 min and the enzyme reaction was terminated with the addition of HCl (200 μl, 2N). The acid metabolites formed ([^{14}C]-5-HIAA and [^{14}C]-PAA) were extracted into toluene (6 ml) by mixing for 3 min and centrifuging at 1,000g for 5 min. The tubes were stored at -80°C for not less then 1 h to freeze the aqueous (bottom) layer. The toluene (top) layer was decanted into vials containing scintillation fluid (9 ml). Radioactive content was determined with the liquid scintillation spectrophotometer. The amount of radioactivity in blank tubes was subtracted from all samples.

E.2 Analysis of Levels of NA, DA, 5-HT, 5-HIAA, HVA, DOPAC and Tryptophan in Rat Brain Using HPLC with Electrochemical Detection (HPLC-EC)

Tissues were homogenized in 5 vols of ice-cold $HClO_4$ (0.1 M) containing Na_2EDTA (10 mg%) and ascorbic acid (0.05 mM). The homogenate was centrifuged at 12,000g for 15 min (4°C) to remove the precipitated protein. The clear supernatant was decanted and directly injected onto the HPLC-EC system described in section B.2. A standard curve containing known amounts of the neurochemicals and their metabolites was prepared and run simultaneously with the samples in order to allow identification of the peaks of interest and their quantification.

E.3 Analysis of Levels of GABA and Other Amino Acids

A gas chromatographic (GC) assay developed by Wong *et al.* (1990a) was used for the simultaneous analysis of GABA, ALA, glycine, valine, leucine and isoleucine concentrations in tissue samples. This involves the sequential derivatization of the amino acids with isobutyl chloroformate and pentafluorophenol, followed by extraction and quantification. Tissues were homogenized as in E.2 with

$HClO_4$ (0.1 μM) containing Na_2EDTA (10 mg%) and ascorbic acid (0.05 μM), and centrifuged at 12,000g for 15 min (4°C). An aliquot (10 μl) of the clear supernatant was then used for the analysis. Norleucine (0.25 μg) was added as an internal standard to the supernatant; this was followed by the addition of potassium carbonate solution (1 ml of 2.5% w/v). Isobutyl chloroformate solution (1 ml of 5 μl isobutyl chloroformate in 1 ml of acetonitrile:toluene, 1:9 v/v) was added to the mixture, which was shaken on a vortex mixer for 15 min (room temperature). After a brief centrifugation, the top (organic) layer was aspirated and discarded. To the bottom (aqueous) phase was added sodium phosphate buffer (1.5 ml, 2M, pH=5.3). This was followed by the sequential addition of chloroform (2.5 ml), dicyclohexylcarbodiimide (DCC) solution (200 μl of 5 μl DCC in 1 ml chloroform) and pentafluorophenol solution (200 μl of 5 μl pentafluorophenol in 1 ml chloroform). These solutions were vortexed for 15 min (room temperature). After a brief centrifugation, the top (aqueous) layer was aspirated and discarded. The bottom chloroform layer was then evaporated (at 40°C) to dryness under a stream of nitrogen. The residue was reconstituted in toluene (300 μl), which was then briefly washed with distilled water (0.5 ml). An aliquot (1 μl) of this toluene layer was used for GC analysis. Chromatographic separation was accomplished using the following automatic temperature program: initial temperature of 100°C for 0.5 min, increasing to 200°C at a rate of 25°C/min; after remaining at 200°C for 0.5 min, the temperature increased at a rate of 3°C/min to a final temperature of 230°C. A standard curve containing known amounts of the amino acids and the internal standard was prepared and run simultaneously with the samples.

E.4 Determination of ALA- and GABA-Transaminase Activity

ALA- and GABA-transaminase (ALA-T and GABA-T) activities were measured with a modification of the procedure of Sterri and Fonnum (1978) in which [^{3}H]-GABA and [^{3}H]-ALA are incubated with tissue homogenates and the anionic products, [^{3}H]-succinate (GABA-T) and [^{3}H]-lactate + [^{3}H]-pyruvate (ALA-T) are extracted and quantified. Tissues were homogenized in 10 vols of a medium described by Palfrymen *et al.* (1978) and consisting of glycerol (20% v/v), Triton X-100 (0.13% v/v), reduced glutathione (100 μM), pyridoxal 5'-phosphate (1 μM), Na_2EDTA (1 mM), K_2HPO_4 (5 mM), and sufficient acetic acid to bring the pH value to 7.2-7.4. An aliquot (5 μl for GABA-T and 10 μl for ALA-T) of the homogenate was added to 20 μl of incubation medium in 1.5 ml microfuge tubes placed on ice. Distilled water was used for blanks instead of tissue. The GABA-T incubation mixture consisted of [^{3}H]-GABA (1 μl), GABA (7.5 μl, 100 mM), α-ketoglutarate (15 μl, 50 mM), nicotinamide adenosine dinucleotide (NAD) (15 μl, 10 mM), 2-aminoethylisothiouronium bromide (15 μl, 10 mM), distilled water (60 μl) and TRIS buffer (37.5 μl, 1 μM, pH=7.9). The ALA-T incubation mixture was modified by adding [^{3}H]-ALA (1 μl) and ALA (7.5 μl, 100 mM) instead of GABA and substituting NAD by NADH (15 μl, 10 mM). The microfuge tubes were then incubated at 37°C for 30 min, before the addition of the liquid anion exchanger tri-*n*-octylamine (TOA) (100 μl of 2.2 ml TOA in 22.8 ml ethyl acetate). The mixture was vortexed briefly, followed by centrifugation at 1,000 g for 2 min. An aliquot (35 μl) of the top layer was removed and added to a counting vial containing scintillation fluid (4 ml). Radioactivity was measured in a liquid scintillation spectrometer after allowing the samples to sit overnight.

E.5 Determination of GAD Activity

GAD activity was measured using a modification of the procedure of Albers and Brady (1959) in which [^{14}C]-glutamic acid is incubated in tissue homogenates and the liberation of [^{14}C]-CO_2 quantified. The homogenization medium and dilution (10 vol) was the same as used in the GABA-T and ALA-T assays (Methods, E.4). The incubation medium contained the following: [^{14}C]-glutamic acid (20 μl), potassium phosphate buffer (10 l, 1M, pH=6.5), dithiothrietol (5 μl, 10 mM), sodium glutamate (5 μl, 500 mM) and distilled water (60 μl). To glass tubes on ice were added incubation medium (10 μl) and brain homogenate (5 μl) (5 μl distilled water to blanks). The glass tubes were connected by 7 cm lengths of polyethylene tubing to a second set of glass tubes containing 2.5 cm Whatman no. 1 filter papers and the solubilizing agent Protosol® (150 μl). The connected tubes were incubated at 37°C for 30 min. The reaction was terminated by the addition of sulfuric acid (50 μl of 6N), and the tubes left to incubate another 40 min. Following the incubation, the glass tubes containing the filter papers were placed in vials containing scintillation fluid (9 ml) and glacial acetic acid (3 drops). Radioactivity was counted in a liquid scintillation spectrometer after allowing the samples to sit overnight.

E.6 Analysis of *p*-OH-PLZ Levels

A modification of the procedure reported by Coutts *et al.* (1980) for analysis of *p*-tyramine was employed. The samples were basified with solid sodium bicarbonate and acetylated by adding acetic anhydride (300 μl) as described by Martin and Baker (1977). After transferring to another set of tubes (leaving behind excess salt), the samples were extracted for 5 min with ethyl acetate (4 ml), and centrifuged for 5 min on a benchtop centrifuge to separate the layers. The organic layers were retained and to each of these layers was added ammonium hydroxide solution (500 μl, 10 N). After mixing for 50 min, the aqueous layers were neutralized with HCl (6 N), and the mixtures were centrifuged to separate the layers. The organic layers were retained and taken to dryness under a stream of nitrogen. The residues were derivatized by adding ethyl acetate (25 μl) and pentafluoropropionic anhydride (75 μl) and heated for 1.5 h at 60°C. At the end of this period, the samples were left at room temperature for 5 min. To each was added toluene (300 μl) and sodium borate buffer (3 ml). The samples were mixed for 5 sec and centrifuged rapidly. An aliquot (1 μl) of the organic layer was injected on a gas chromatograph equipped with an electron-capture detector. A calibration curve consisting of varying concentrations of *p*-OH-PLZ and a fixed amount of internal standard (*p*-chlorophenylethylamine) was handled in parallel with each assay run. The procedure described above was used for analysis of free *p*-OH-PLZ. In order to measure total (free + conjugated) *p*-OH-PLZ, samples were first boiled for 60 min after adding HCl (100 μl, 6 N) and then carried through the procedure described above. The assay procedure and derivatization steps are summarized in Figure 10.

E.7 Confirmation of Structure of *p*-OH-PLZ using GC-MS

Mass spectra (for confirmation of structures of the derivaties) were recorded in the electron-impact mode using a VG 7070E mass spectrometer (VG Instruments, Manchester, UK) linked to a Varian Vista gas chromatograph (Varian

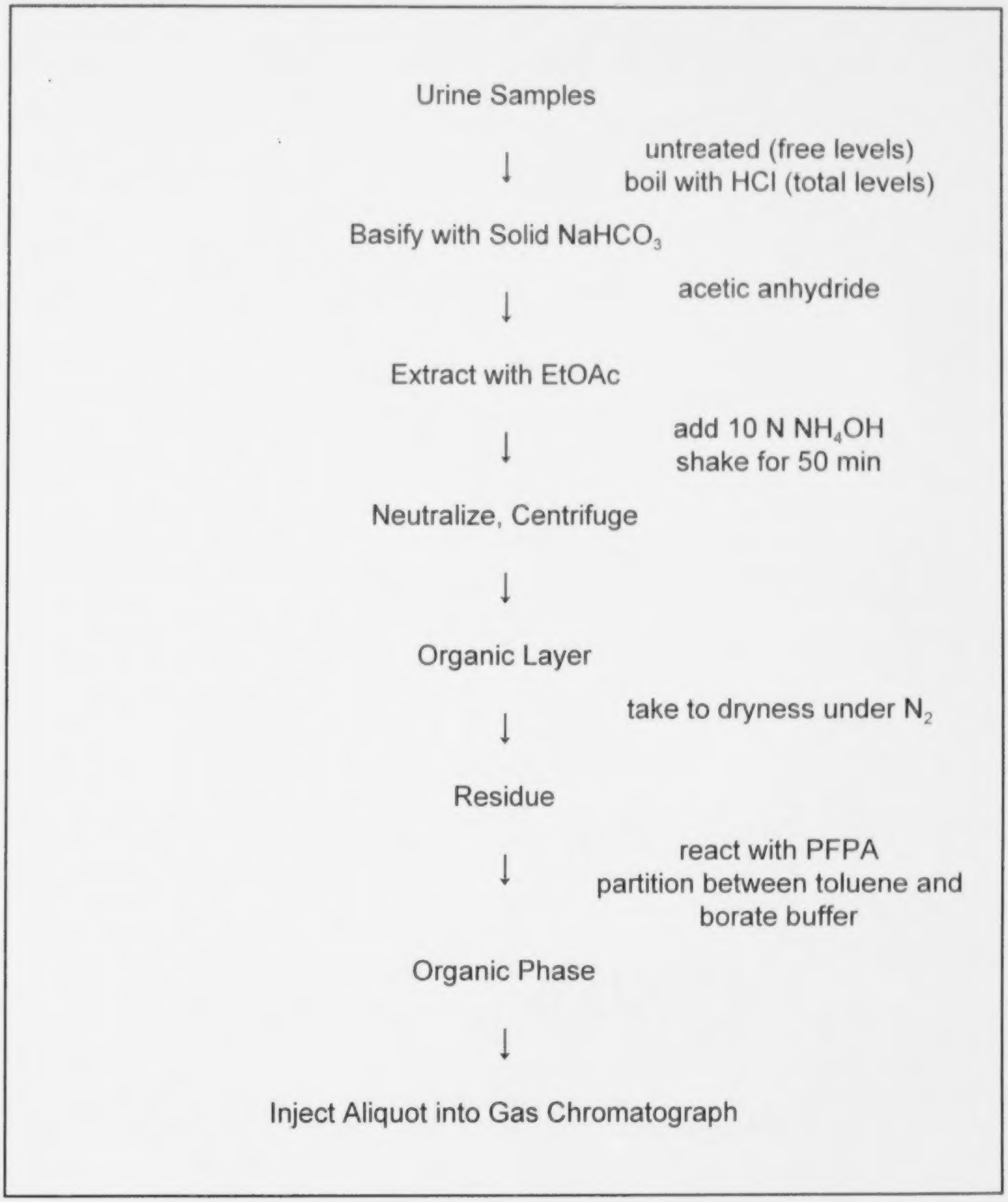
Urine Samples
untreated (free levels)
boil with HCl (total levels)
Basify with Solid $NaHCO_3$
acetic anhydride
Extract with EtOAc
add 10 N NH_4OH
shake for 50 min
Neutralize, Centrifuge
Organic Layer
take to dryness under N_2
Residue
react with PFPA
partition between toluene and
borate buffer
Organic Phase
Inject Aliquot into Gas Chromatograph

Figure 10: Summary of the assay procedure for *p*-OH-PLZ.

Instruments, Sunnyvale, CA, USA) containing a 30 m x 0.25 mm I.D. DG-5 (0.22 μm film thickness; J & W Scientific) fused-silica capillary column. The temperature program used for separation was the same as used for the GC assay described in section E.6 above.

E.8 Analysis of PLZ and PEA Levels in Rat Brain

To measure rat brain concentrations of PLZ and PEA, an electron-capture gas chromatographic method following extraction and derivatization with pentafluorbenzoyl chloride (PFBC) was used (Rao *et al.*, 1987). Rat brains were homogenized in 5 vol of ice-cold $HClO_4$ (0.1 μM) containing Na_2EDTA (10 mg%) and ascorbic acid (0.05 mM), followed by centrifugation at 12,000g for 15 min (4°C to remove the precipitated protein. Aliquots of the supernatant (3 ml) were retained and internal standard (benzylamine, 200 ng) was added. Excess acid was neutralized with the addition of solid $KHCO_3$, and the resultant precipitate was removed by centrifugation (3,000 μg, 3 min). The clear supernatants were transferred to another set of tubes, phosphate buffer (300 μl, 0.1 μM, pH=7.8) was added, and the solution extracted by shaking for 5 min with the liquid ion-pairing agent di-(2-ethylhexyl)phosphate (DEHPA, 3 ml of 2.5 % v/v in chloroform). Following a brief centrifugation, the upper (aqueous) layer was aspirated off, and the organic layer was back-extracted with HCl (2.5 ml, 0.5 N). The samples were centrifuged briefly to separate the phases, and the aqueous layers were transferred to another set of tubes and basified with solid $KHCO_3$. A solution of toluene:acetonitrile:PFBC (3 ml, 9:1:0.015) was added, and the tubes were shaken vigorously for 10 min. A further 2 μl of PFBC in toluene (20 μl) was added and shaking was continued vigorously for 10 min, after which the samples were centrifuged. The organic layers were transferred to another set of tubes and taken to dryness under a stream of nitrogen. The residues were were taken up in toluene (300 μl) and washed rapidly with ammonium hydroxide (500 μl, 1.0 N). An aliquot (1 μl) of the toluene layer was injected on the gas chromatograph for analysis. A standard curve containing known amounts of PLZ and PEA was run simultaneously with tissue samples to allow quantification.

E.9 Analysis of MHPG Levels in Human Subjects

Urinary MHPG levels were quantitated using acetylation to extract MHPG into ethyl acetate followed by derivatization with trifluoroacetic anhydride under anhydrous conditions (Baker *et al.*, 1987a). To 2 ml portions of the 24 h urine samples were added sodium acetate solution (0.66 ml, 1 N, pH=6), β-glucuronidase/aryl sulfatase (glusalase, 0.066 ml), and internal standard (*p*-hydroxybenzoyl alcohol, 6 μg). The pH was adjusted to a value of 6.0, and the mixtures were incubated for 24 h at 37°C. Portions (1.0 ml) were then added to 1.5 ml microfuge tubes on ice. $HClO_4$ (100 μl, 4 N) was added , the mixtures vortexed and centrifuged briefly, and the resultant supernatants were transferred to a set of screw-cap glass tubes. The samples were neutralized by the addition of solid potassium bicarbonate, and centrifuged at 1,000 g for 5 min. The resultant supernatants were transferred to another set of tubes and acetic anhydride (200 μl) was added. Acetylation was continued for 20 min, with solid sodium bicarbonate being added so that a small excess was maintained at the bottom of the tubes (Martin and Baker, 1977). The supernatants were transferred to another set of tubes and ethyl acetate (4.5 ml) was added. The tubes were shaken for 2 min and centrifuged for 5 min at 1,000 g. The ethyl acetate phases were transferred to another set of tubes and evaporated to dryness under a stream of nitrogen. Ethyl acetate (25 μl) and trifluoroacetic anhydride (75 μl) were added to each tube and the tubes were left standing 30 min (room temperature). Cyclohexane (300 μl) and saturated sodium tetraborate solution (3.0 ml) were added and the tubes were vortexed and centrifuged briefly. Aliquots (1 μl) of the cyclohexane phase were analyzed using GC with an electron capture detector. A set of standards of known concentrations of MHPG was run in parallel with each assay in order to prepare a calibration curve for quantitative purposes.

E.10 Analysis of Urinary Levels of *m*- and *p*-OH-PAA, HVA and 5-HIAA in Human Subjects

The procedure described by Baker *et al.* (1987b) was employed. To an aliquot (0.5 ml) of the 24 h urine sample containing internal standard (*p*-hydroxyphenylpropionic acid) were added HCl (100 μl, 1 N), NaCl (675 mg) and chloroform (5 ml). The samples were shaken for 5 min and centrifuged (1,000 g for 5 min) to separate the phases. The organic layers were taken to dryness with nitrogen and the residues dissolved in ethyl acetate (25 μl). Pentafluoropropionic anhydride (50 μl) was added, and the tubes were left at 60°C for 25 min and cooled at room temperature for 10 min. Hexafluoroisopropanol (100 μl) was added, and the tubes left standing at 60°C for another 35 min. The samples were partitioned between toluene (1 ml) and sodium phosphate buffer (3.0 ml, 0.25 M, pH=6.0) for 15 sec, and the phases separated by centrifugation (1,000g) for 1 min. An aliquot (1 μl) of the organic phase was used for injection on the gas chromatograph. Solutions of known, varying amounts of the authentic standards of the acids were carried through the procedure in parallel with the samples to allow quantification.

E.11 Analysis of Urinary Levels of 3-MT, NME, *m*-TA, *p*-TA, Tryptamine and 5-HT in Human Subjects

Analyses were conducted as described by Coutts *et al.* (1981) and Baker *et al.* (1981). To a 4 ml aliquot of each 24 h urine sample, internal standards (3-phenylpropylamine or 5-methyltryptamine, 500 ng) were added, the pH value was adjusted to 7.8 and sodium phosphate buffer (400 μl, 0.25 M, pH=7.8) was added. The samples were shaken for 1 min with the liquid anion exchanger DEHPA (5 ml, 2.5% v/v in chloroform) and, following brief centrifugation, the upper (aqueous) layers were aspirated. The organic layer in each case was shaken with HCl (2.5 ml, 0.5 N) for 2 min. After centrifuging, the HCl phases were retained and neutralized with solid sodium bicarbonate. Samples were then acetylated with acetic anhydride (250 μl) and extracted with ethyl acetate (4 ml). In each case, the ethyl acetate layer was divided into two portions: 1 ml for analysis of 5-HT and tryptamine, and 3 ml for analysis of *m*-TA, *p*-TA, NME and 3-MT. The 1 ml portion was taken to dryness under nitrogen, reacted with pentafluoropropionic anhydride and analyzed on the gas chromatograph (Baker *et al.*, 1981). To the 3 ml portion of ethyl acetate was added ammonium hydroxide solution (400 μl, 10 N). This was shaken for 40 min to hydrolyze acetylated phenolic groups, the aqueous layer was neutralized with HCl (6N), and the organic phase retained and taken to dryness under nitrogen. The residue was reacted with trifluoroacetic anhydride (75 μl) in the presence of ethyl acetate (25 μl) at room temperature for 30 min (Coutts *et al.*, 1980). The reaction mixture was partitioned between cyclohexane (300 μl) and saturated sodium borate buffer (3.0 ml). An aliquot (1 μl) of the cyclohexane phase was injected on the gas chromatograph for analysis.

E.12 Analysis of Urinary Levels of PAA in Human Subjects

Levels of PAA, a major metabolite of PEA and PLZ, were measured using gas chromatography following derivatization under aqueous conditions (Wong *et al.*, 1988). A portion (25 μl) of the 24 h urine sample was added to test tubes containing internal standard (p-Cl-PAA). An equal volume of HCl (25 μl, 6 M) was added to each and the tubes left in a boiling water bath for 60 min. After cooling, the volume in each tube was made up to 200 μl with water before the addition of sodium phosphate buffer (2 M, pH=6.0). After brief mixing, toluene (200 μl), dicyclohexylcarbodiimide (DCC) solution (200 μl of 5 μl DCC/ml toluene-acetonitrile [9:1 v/v]), and pentafluorophenol (PFPh-OH) solution (200 μl of 5 μl PFPh-OH/ml toluene-acetonitrile [9:1 v/v]) were added sequentially. The two phases were shaken for 15 min. After a brief centrifugation, the organic layers were transferred to 1.5 ml microfuge tubes containing ammonium hydroxide (400 μl, 0.5 M). The tubes were vortexed for 5 sec and centrifuged briefly to separate the phases. The organic phases were retained for GC analysis. A standard curve containing known amounts of PAA and the same amount of internal standard as in the samples was processed in parallel with the samples to allow quantification.

E.13 Analysis of Urinary Levels of PEA in Human Subjects

Analysis of PEA was conducted using the extractive derivatization procedure described by Baker *et al.* (1986). Portions (4 ml) of the 24 h urine sample, with added internal standard [2-(4-chlorophenyl)ethylamine, 500 ng], were basified by addition of solid potassium hydrogen carbonate and centrifuged briefly at 1,000g for 3 min. The clear supernatants were transferred to another set of tubes, sodium phosphate buffer (400 l, 0.1 M, pH= 7.8) was added to each tube, and the urines were extracted by shaking with chloroform (4 ml) containing the liquid ion-pairing reagent DEHPA (2.5 % v/v). Following brief centrifugation, the top aqueous layer was aspirated off and the chloroform layer was shaken vigorously with HCl (2.5 ml, 0.5 M) for 5 min. The aqueous acid layer was transferred to another set of tubes following brief centrifugation. The supernatants were basified with the addition of solid potassium carbonate, and derivatized with a mixture of toluene-acetonitrile-pentafluorobenzenesulfonyl chloride (4 ml, 9:1:0.01). The tubes were shaken vigorously for 2 min and, following a brief centrifugation, the top (organic) layers were transferred to a clean set of tubes and taken to dryness under a stream of nitrogen. The residue was taken up in toluene (300 μl) and an aliquot (1 μl) was used for GC analysis. Standard curves consisting of varying amounts of authentic PEA and the same amount of internal standard as added to the patient samples were run in parallel with each assay run.

E.14 [^{3}H]-Muscimol Binding Assay

[^{3}H]-Muscimol was used as the radioligand to determine the number (B_{max}) and affinity (K_D) of GABA$_A$ binding sites in rat brain homogenates according to a modification of the procedure described by Ito *et al.* (1988). To prepare the membranes, rat brain cortex was homogenized in ice-cold 0.32 M sucrose (10 vol) and centrifuged at 1,000 g for 10 min. The nuclear pellet was discarded after the supernatant was collected. The supernatant was then centrifuged at 20,000 g for 26 min at 4°C . The pellet was resuspended in ice-cold double distilled-water (10 vol) and centrifuged at 8,000 g for 24 min (4°C). The pellet was washed a further 2 times with 9 vol of ice-cold water and each time centrifuged at 48,000 g for 27 min (4°C). Following the final centrifugation the pellet was stored at -20°C overnight. The next day the pellet was resuspended in 10 vol of ice-cold 50 mM TRIS buffer with 50 mM NaCl (pH = 7.1 with citric acid) and centrifuged at 48,000 g for 27 min (4°C). The pellet was resuspended in 40 vol of the TRIS buffer and incubated at 37°C for 30 min. TRIS buffer was then added to bring the total volume to 40 ml and this was centrifuged at 25,000 g for 28 min (4°C). The resultant pellet was resuspended in 20 vol buffer to give a final membrane suspension containing approximately 0.18 to 0.26 mg protein per ml.

For the binding assay, aliquots (200 μl) of the final membrane suspension were incubated for 60 min at 0°C in tubes containing [^{3}H]-muscimol (20 Ci/mmol) (5 nM) and one of 6 concentrations of unlabelled muscimol (2-64 nM). Buffer was added to a final volume of 1 ml. Non-specific binding was defined using unlabelled GABA (1 mM) and represented approximately 10-20% of total binding. The incubation was terminated by rapid filtration. Filters were washed 3 times with approximately 4 ml ice-cold buffer per wash. Following filtration, the filters were placed in vials containing scintillation fluid (5 ml) and allowed to sit for 12 h before counting in a liquid scintillation spectrometer.

E.15 [^{3}H]-Flunitrazepam Binding Assay

[^{3}H]-Flunitrazepam was used to define the K_D and B_{max} of benzodiazepine binding sites in rat cortex and hippocampus according to the procedure described in Kimber *et al.* (1987). Whole cortex or hippocampus was homogenized in 20 or 40 vol respectively of ice-cold 50 mM TRIS buffer (pH=7.1) and centrifuged at 40,000 g for 20 min (4°C). The pellet was resuspended in the original volume of TRIS buffer and centrifuged at 40,000 g for 20 min 5 more times for a total of 6 washes. Following the sixth wash, the pellet was was resuspended in TRIS buffer and aliquots were frozen at -20°C. On the day of the assay, the frozen suspension was thawed and centrifuged at 40,000 g for 20 min (4°C). The pellet was resuspended in 80 vol of TRIS buffer and used for binding to give a final membrane suspension containing about 0.08-0.10 mg protein per ml.

Routine binding assays were carried out using 100 μl of tissue suspension incubated for 60 min at 0°C with appropriate drug solutions and 50 mM TRIS buffer to make a final volume of 1 ml. [^{3}H]-Flunitrazepam at 5 concentrations ranging from

0.25 to 7.5 nM was used as the hot ligand and nonspecific binding was defined using clonazepam (3 μM). To examine GABA-facilitation of [^{3}H]-flunitrazepam binding, GABA (10^{-4} M) and NaCl (150 mM) were added to a series of tubes. The incubation was terminated by rapid filtration, followed by washing 3 times with approximately 4 ml ice-cold buffer per wash. Following filtration, the filters were placed in vials containing scintillation fluid (5 ml) and allowed to sit for 12 h before counting in a liquid scintillation spectrometer.

E.16 Functional Testing of α_2-Adrenergic Receptors

A functional assessment of α_2-adrenergic autoreceptors was conducted using the α_2-agonist clonidine (Greenshaw *et al.*, 1988). The motor suppressant effects of clonidine were examined on days 21 and 22 of chronic administration of PLZ and N^{2}AcPLZ. On each day either clonidine hydrochloride (50 μg/kg) or the 0.9% saline vehicle was injected i.p. 15 min prior to behavioural testing. At this dose clonidine is a selective presynaptic α_2-adrenergic receptor agonist. Each animal received both saline and clonidine on alternate days. A randomly selected half of each treatment group received an injection of clonidine on day 21 followed by saline on day 22. In the other animals the order of injections was reversed. The animals were individually placed in computer-controlled infrared activity measurement systems for a period of 30 min under reduced lighting conditions in a quiet environment.

E.17 Determination of the Inhibition of Uptake of Neurotransmitter Amines from Brain Tissue

Following sacrifice of a control rat (Methods C.2), the corpus striatum (for DA or 5-HT) or hypothalamus (for NA) was dissected out and chopped into prisms (0.1 x 0.1 x 2 mm) with a tissue chopper. The tissues were dispersed in ice-cold incubation medium consisting of NaCl (123 mM), KCl (5 mM), $CaCl_2$ (2.7 mM), $MgSO_4$ (1.2 mM), glucose (10 mM), ascorbic acid (1 mM), the MAO inhibitor nialamide (12.5 μM) and TRIS buffer (20 mM, pH=7.4) so that a final tissue concentration of 5 mg/ml was obtained.

The tissue suspensions (1 ml) were added to 25 ml flasks containing 4 ml of incubation mix (blanks contained 5 ml incubation mix). The flasks were incubated in a water bath for 15 min at 37°C. The drug solutions were then added to the appropriate flasks, followed by the addition of the radiolabelled substrate mixture (final concentration 0.02 μM). The flasks were incubated for 5 min at 37°C, after which the contents of each were placed on a filter paper in a receptacle well of the Millipore filtration apparatus with the vacuum on. Each flask was rinsed twice with warm (37°C) incubation mix (5 ml) and the rinses poured through the filtration apparatus. The filters were removed and placed in vials containing scintillation fluid (10 ml) and counted in a liquid scintillation counter. The blanks were subtracted from all samples, and then the drug-treated samples were expressed as a percentage of control values. These values were subtracted from 100 to express the data as a percent uptake inhibition.

IC_{50} values were determined by plotting % inhibition versus drug concentration on semilog graph paper. At least 5 experiments were used for determination of the IC_{50} value in each case.

E.18 Determination of the Release of Neurotransmitter Amines from Brain Tissue

The tissue suspensions were prepared as in Methods E.16 to give a final tissue concentration of 1 mg/ml of incubation mix. The prisms were prepared from tissue dissected from the corpus striatum for DA and 5-HT experiments and hypothalamus for NA experiments.

The tissue suspensions (1 ml) were preincubated for 10 min at 37°C, at which time the radiolabelled neurotransmitter was added (final concentration 0.02 μM). The samples were incubated for a further 10 min at 37°C and placed on a Millipore filter (#40) attached to a vacuum line in the tissue chamber of the superfusion apparatus (Methods B.6). After rapidly washing twice with incubation medium at 37°C, the tissues were superfused with incubation mix at a rate of 0.5 ml/min and superfusates were collected each successive 1 min. After 4 min, the medium in some of the chambers was replaced with medium containing varying concentrations of the drugs of interest and 5 further fractions were collected. To each fraction was added scintillation fluid (5.5 ml): the amounts of radioactivity were measured by counting with a liquid scintillation counter.

E.19 Determination Of Protein Concentrations

Protein concentrations in the rat brain homogenates used in binding studies were determined according to the procedure of Lowry *et al.*, (1951). To an aliquot (50 μl) of brain homogenate were added distilled water (750 μl) and membrane digestor (200 μl, 1 N sodium hydroxide-1% sodium deoxycholate [1:1 v/v]). The mixture was vortexed, incubated for 10 min (room temperature), and reagent A (5 ml, 2% sodium carbonate-1% cupric sulfate-2% sodium potassium tartrate [1/0.01/0.01 v/v/v]) was added. The tubes were vortexed, incubated for a further 10 min, following which folin reagent was added (500 μl, 1N folin) and the tubes vortexed and incubated for 30 min. A standard curve containing known amounts of bovine serum albumin was run in parallel with the tissue samples. All tubes were analyzed with a spectrophotometer set at a wave length of 660 nm.

F. STATISTICAL ANALYSIS

Statistical analysis consisted of analysis of variance (one- or two-way ANOVA) followed by independent t-tests in the case of single pair comparisons or Newman-Keuls tests in the case of multiple comparisons. Data from radioligand binding assays was analyzed using EBDA (Equilibrium Binding Data Analysis) and LIGAND programs (McPherson, 1987) to determine the binding parameters, B_{max} (density of binding sites) and K_D (dissociation constant). Data from human experiments were analyzed using a repeated measures ANOVA followed by Newman-Keuls tests when appropriate. Locomotor activity data in the α_2-adrenergic functional probe were analyzed with nonparametric statistical methods which involved a Kruskal-Wallis ANOVA followed by Mann-Whitney U-tests.

Statistical significance was designated by * $P<0.05$ or ** $P<0.01$. In certain graphs a mean SEM derived from the Mean Square error term is indicated. This represents the average SEM and is calculated following the ANOVA by taking the square root of the mean square error divided by the harmonic n.

RESULTS

A. EFFECTS OF PHENELZINE AND N^2ACETYLPHENELZINE IN THE RAT

A.1 MAO Activity

A.1.1 Effects of Acute Administration of PLZ and N^2AcPLZ

Both PLZ and N^2AcPLZ significantly inhibited MAO-A and -B at all times and doses studied. The dose response and time course studies of MAO inhibition *ex vivo* are shown in Tables 8 and 9. Phenelzine is a more potent MAO inhibitor than is N^2AcPLZ, causing significantly greater inhibition of MAO-A and MAO-B at doses of 0.05 and 0.10 but not 0.20 mmol/kg, and at 1 and 2 but not 4 and 8 h. Both drugs display a tendency to inhibit MAO-A more than MAO-B, an effect which is more apparent at lower doses.

A.1.2 Effects of Chronic Administration of PLZ and N^2AcPLZ

The effects of chronic (28 d) PLZ and N^2AcPLZ drug treatment on brain, liver and heart MAO activity are shown in Table 10. Both drugs caused more than 85% inhibition of MAO-A and -B after 28 days of administration. The drugs did not display selectivity towards either MAO-A or MAO-B.

A.1.3 Effects of PLZ and N^2AcPLZ *In Vitro*

MAO activity was analyzed *in vitro* by adding various concentrations of PLZ and N^2AcPLZ to control brain homogenates and incubating 10 min prior to adding the radiolabelled substrates (Methods E.1). Percentage inhibition versus logarithm

Table 8: Inhibition of MAO *ex vivo* at 1 h after administration of PLZ or N^2AcPLZ (0.05, 0.10 and 0.20 mmol/kg i.p.). Results are expressed as mean % inhibition (SEM) compared to control values, n=6. Control MAO-A activity = 70.3 ± 3.9 pmol/min/mg tissue, n=18. Control MAO-B activity = 67.5 ± 2.0 pmol/min/mg tissue, n=18. ** P<0.01 compared to control values.

Dose (mmol/kg)	MAO-A		MAO-B	
	PLZ	N^2AcPLZ	PLZ	N^2AcPLZ
0.05	97.9 ± 0.5**	40.5 ± 2.3**	70.8 ± 3.2**	21.3 ± 1.6**
0.1	96.9 ± 0.4**	74.6 ± 6.0**	78.4 ± 2.3**	54.6 ± 5.1**
0.2	97.8 ± 0.3**	94.9 ± 2.5**	87.2 ± 0.9**	79.6 ± 4.0**

Table 9: Inhibition of MAO *ex vivo* at 1, 2, 4 and 8 h after administration of PLZ or N^2AcPLZ (0.10 mmol/kg i.p.). Results are expressed as mean % inhibition (SEM) compared to control values, n=4-6. Control MAO-A activity = 68.2 ± 3.3 pmol/min/mg tissue, n=23. Control MAO-B activity = 62.9 ± 2.4 pmol/min/mg tissue, n=24. ** $P<0.01$ compared to control values.

Time	MAO-A		MAO-B	
(h)	PLZ	N^2AcPLZ	PLZ	N^2AcPLZ
1	96.9 ± 0.4**	74.6 ± 6.0**	78.4 ± 2.3**	54.6 ± 5.1**
2	98.7 ± 0.2**	87.5 ± 2.5**	85.2 ± 2.3**	61.9 ± 5.7**
4	96.4 ± 0.5**	93.4 ± 0.7**	77.3 ± 5.4**	74.1 ± 1.6**
8	90.0 ± 4.2**	95.8 ± 1.1**	74.1 ± 12.0**	80.7 ± 2.2**

Table 10: Effects of chronic (28 d) phenelzine (0.05 mmol/kg/d) and N^2AcPLZ (0.10 mmol/kg/d) administration on brain, liver and heart MAO activity. Results are expressed as mean % inhibition of MAO ± SEM compared to control values, n=10.

	BRAIN		LIVER		HEART	
	MAO-A	MAO-B	MAO-A	MAO-B	MAO-A	MAO-B
PLZ	90 ± 2	89 ± 1	90 ± 2	91 ± 1	99 ± 0.3	94 ± 2
N^2AcPLZ	91 ± 0.7	89 ± 1	93 ± 1	98 ± 0.2	98 ± 0.4	95 ± 2

of concentration plots on semilogarithm graph paper were used to determine the concentration of each drug required to cause 50% inhibition of MAO-A or MAO-B. Each experiment was conducted 6 times.

Phenelzine is 40 times more potent then N^2AcPLZ at inhibiting MAO-A [F(1,10)=52.8, ($P<0.01$)] and 90 times more potent at blocking MAO-B *in vitro* [F(1,9)=16.8, ($P<0.01$)]. The concentration of drug (M) needed to cause 50% inhibition of MAO (IC_{50}) is shown in Table 11. Both PLZ [F(1,10)=6.3, ($P<0.05$)] and N^2AcPLZ [F(1,9)=8.0, ($P<0.05$)] were slightly more potent at inhibiting MAO-A than MAO-B *in vitro*.

A.2 Effects on Levels of Biogenic Amines and Metabolites

A.2.1 Effects of Acute Administration of PLZ and N^2AcPLZ

Phenelzine significantly elevated whole brain levels of NA, DA and 5-HT at all times and doses studied, whereas higher doses of N^2AcPLZ were required to significantly elevate whole brain levels of NA, DA (0.20 mmol/kg) and 5-HT (0.10 mmol/kg) at 1 h (Figure 11). In the time course study, N^2AcPLZ (0.10 mmol/kg) significantly raised 5-HT at 1, 2, 4 and 8 h, and NA and DA at 2, 4 and 8 h (Figure 12). It was observed that PLZ elevated NA and 5-HT levels significantly more than equimolar doses of N^2AcPLZ at 1 h, but by 8 h post drug administration (0.10 mmol/kg) there were no significant differences in the effects of PLZ and N^2AcPLZ on NA, 5-HT or DA levels. Both drug treatments induced decreases in whole brain levels of the acid metabolite DOPAC, but higher doses of N^2AcPLZ were required to induce significant decreases in HVA (0.10 mmol/kg) and 5-HIAA (0.20 mmol/kg)

Table 11: Inhibition of MAO *in vitro* by PLZ and N^2AcPLZ. Results expressed as concentration of drug (M) needed to cause 50% inhibition of MAO ± SEM, n=6 (except N^2AcPLZ -- MAO-B, n=5).

	IC_{50} (M)	
Drug	MAO-A	MAO-B
PLZ	$3.3 \pm 0.33 \times 10^{-9}$	$5.0 \pm 0.60 \times 10^{-9}$
N^2AcPLZ	$1.4 \pm 0.18 \times 10^{-7}$	$4.5 \pm 1.2 \times 10^{-7}$

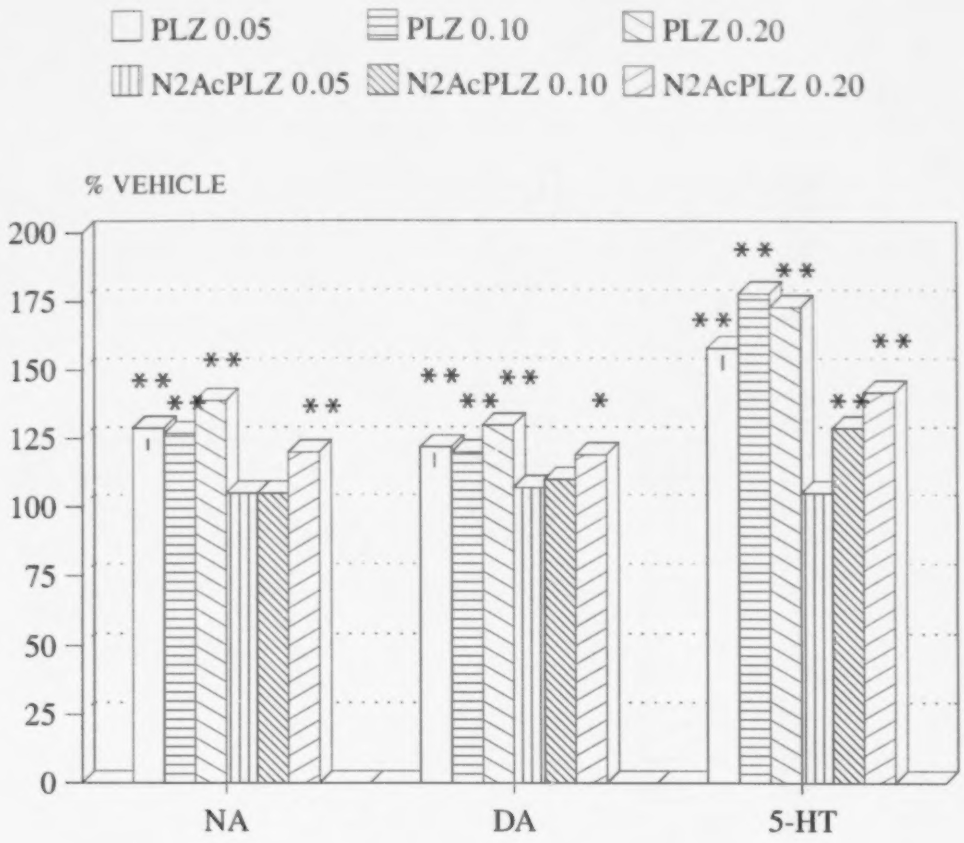

Figure 11: Effects of PLZ and N^{2}AcPLZ (0.05, 0.1 and 0.2 mmol/kg i.p.) on whole brain levels of NA, DA and 5-HT at 1 h. Results expressed as % control, n=6. Control levels: NA = 367 ± 9.0 ng/g; DA = 721 ± 7.9 ng/g; 5-HT = 460 ± 8.7 ng/g; n=18. * $P<0.05$; ** $P<0.01$, values compared to controls. The line in the clear box represents the standard error derived from the MSerror term.

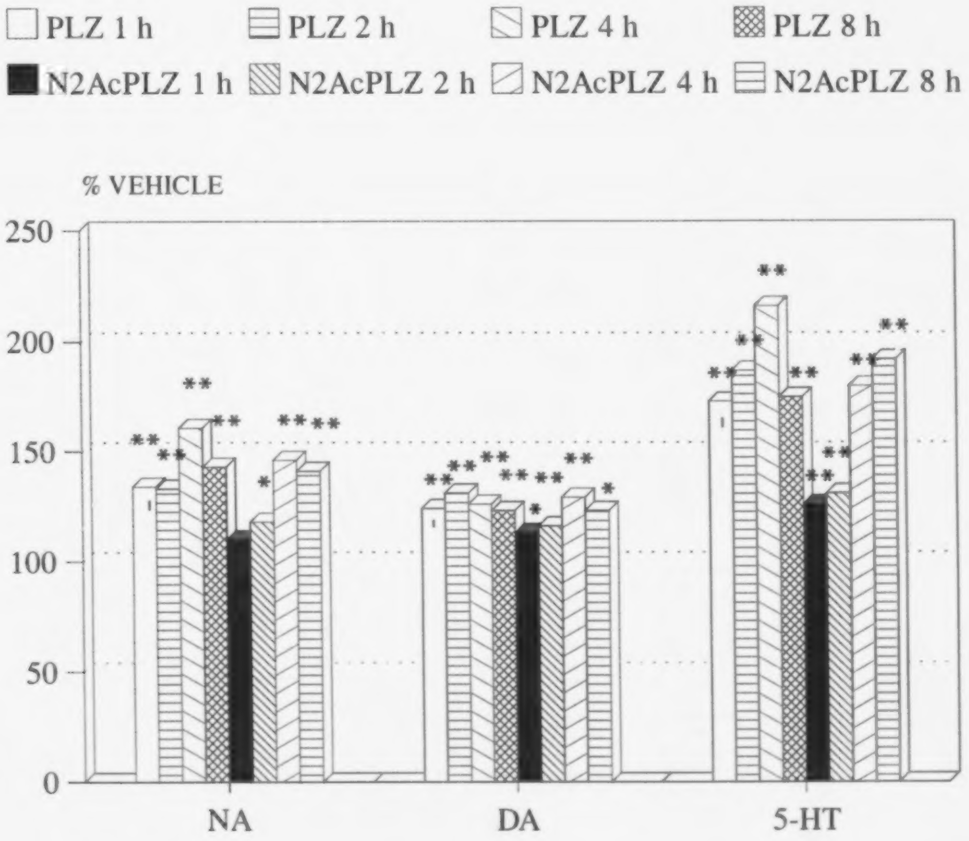

Figure 12: Effects of PLZ and N^{2}AcPLZ (0.1 mmol/kg i.p.) on whole brain levels of NA, DA and 5-HT at 1, 2, 4 and 8 h. Results expressed as % control, n=6. Control levels: NA = 333 ± 6.8 ng/g; DA = 689 ± 10.3 ng/g; 5-HT = 451 ± 9.5 ng/g; n=24. * $P<0.05$; ** $P<0.01$, values compared to controls. The line in the clear box represents the standard error derived from the MSerror term.

(Tables 12 and 13). Phenelzine caused significantly lower levels of 5-HIAA than did N^2AcPLZ (equimolar doses).

A.2.2 Effects of Chronic Administration of PLZ and N^2AcPLZ

Whole brain levels of NA, DA and 5-HT were elevated by chronic (28 d) drug treatment as shown in Table 14. There were no significant differences between PLZ- and N^2AcPLZ-treated rats at the doses studied. Levels of the acid metabolites DOPAC, HVA and 5-HIAA were decreased in the drug-treated groups. As in the acute study, PLZ caused a greater suppression of 5-HIAA levels than did N^2AcPLZ. The effects of PLZ and N^2AcPLZ on amine neurotransmitters were most pronounced in the case of 5-HT (3-fold elevation) and least marked for DA. The suppression of DA metabolites HVA and DOPAC was so pronounced that they were regularly below the threshold of detection (approximately 10 ng/g of brain tissue). In contrast, 5-HIAA formation was inhibited to a lesser degree. As MAO is ubiquitous and probably never completely inhibited, the greatly elevated levels of 5-HT may result in the significant 5-HIAA formation despite high levels of MAO inhibition.

A.3 Effects on γ-Aminobutyric Acid and Other Amino Acids

A.3.1 Effects of Acute Administration of PLZ and N^2AcPLZ

The effects of acute administration of PLZ and N^2AcPLZ (0.05, 0.10 and 0.20 mmol/kg at 1 h and 0.10 mmol/kg at 1, 2, 4, and 8 h) on whole brain levels of GABA

Table 12: Effects of PLZ and N^2AcPLZ (0.05, 0.1 and 0.2 mmol/kg i.p.) 1 h post-injection on whole brain levels of HVA, DOPAC and 5-HIAA. Results expressed as mean concentration (SEM) in ng/g. n=6 (drug-treated groups), n=18 (vehicle). * $P<0.05$; ** $P<0.01$, values compared to controls. n.d. = not detectable (i.e. < 10 ng/g).

Drug	HVA	DOPAC	5-HIAA
Vehicle	82.7 ± 4.7	68.5 ± 2.6	322 ± 8.7
PLZ: 0.05 mmol/kg	n.d.	n.d.	142 ± 15**
PLZ: 0.1 mmol/kg	n.d.	n.d.	150 ± 8**
PLZ: 0.2 mmol/kg	n.d.	n.d.	185 ± 11**
N^2AcPLZ: 0.05 mmol/kg	65.4 ± 4.0	42.6 ± 9.2**	306 ± 8
N^2AcPLZ: 0.1 mmol/kg	55.6 ± 13.6*	24.7 ± 7.5**	282 ± 17
N^2AcPLZ: 0.2 mmol/kg	n.d.	n.d.	254 ± 10**

Table 13: Effects of PLZ and N^2AcPLZ (0.1 mmol/kg i.p.) at 1, 2, 4 and 8 h post-injection on whole brain levels of HVA, DOPAC and 5-HIAA. Results expressed as mean concentration (SEM) in ng/g. n=6 (drug-treated groups), n=24 (vehicle). * P<0.05; ** P<0.01, values compared to controls. n.d. = not detectable.

Drug	HVA	DOPAC	5-HIAA
Vehicle	88.7 ± 2.4	75 ± 2.0	317 ± 7.7
PLZ: 1 h	n.d.	n.d.	150 ± 7.7**
PLZ: 2 h	n.d.	n.d.	142 ± 3.7**
PLZ: 4 h	6.2 ± 6.2**	n.d.	128 ± 9.0**
PLZ: 8 h	40.2 ± 4.4**	22.4 ± 6.6**	252 ± 8.5**
N^2AcPLZ: 1 h	55.6 ± 13.6*	24.7 ± 7.5**	282 ± 16.9
N^2AcPLZ: 2 h	40.7 ± 16.7**	27.3 ± 7.4**	322 ± 8.8
N^2AcPLZ: 4 h	14.9 ± 7.5**	n.d.	216 ± 10.1**
N^2AcPLZ: 8 h	21.3 ± 8.4**	n.d.	252 ± 8.5**

Table 14: Effects of chronic (28 d) PLZ (0.05 mmol/kg/d) and N^2AcPLZ (0.1 mmol/kg/d) administration on whole brain levels of neurotransmitter amines and acid metabolites. Results are expressed as ng/g (mean ± SEM), n=6. ** P<0.01 compared to control values. n.d. = not detectable.

	VEHICLE	PLZ	N^2AcPLZ
NA	375 ± 16	707 ± 39**	729 ± 35**
DA	837 ± 36	1064 ± 36**	1128 ± 22**
5-HT	304 ± 8	946 ± 8**	894 ± 29**
DOPAC	104 ± 6	n.d.	n.d.
HVA	90 ± 10	n.d.	n.d.
5-HIAA	243 ± 11	61 ± 11**	110 ± 7**

and ALA are shown in Figures 13 and 14. At all doses and times studied PLZ induced significant elevations in whole brain levels of GABA compared to vehicle- and N^2AcPLZ-treated animals. Each increase in dose of PLZ caused significantly higher levels of GABA. In marked contrast to PLZ, N^2AcPLZ had no effect on whole brain levels of GABA or ALA. Neither treatment had an effect on brain levels of glycine (GLY), valine (VAL), leucine (LEU) or isoleucine (ILEU). An additional study was performed in which rats (n=5) were administered a very high dose of N^2AcPLZ (0.50 mmol/kg) or vehicle (DMSO at this high dose in order to get the drug into solution) and sacrificed at 2 h post-injection (i.p.). Again, there was no significant effect of N^2AcPLZ on whole brain levels of GABA or ALA compared to the vehicle group.

A.3.2 Effects of Chronic Administration of PLZ and N^2AcPLZ

The effects of chronic (28 d) administration of PLZ and N^2AcPLZ on brain levels of GABA and ALA are shown in Figure 15. PLZ induced sustained elevations in the levels of GABA [$F(2,15)=4.6$; $P<0.05$] and ALA [$F(2,15)=20.7$; $P<0.05$] but to a lesser degree than observed after acute administration. N^2AcPLZ had no effect on levels of GABA and ALA. There was no effect of treatment with either drug on levels of GLY, VAL, or LEU. A significantly lower level of ILEU compared to controls was seen in the N^2AcPLZ treated group [$F(2,15)=4.5$; $P<0.05$]. Mean levels of ILEU in μg/g were 7.7 ± 0.2 (VEH), 7.5 ± 0.4 (PLZ) and 6.6 ± 0.3 (N^2AcPLZ).

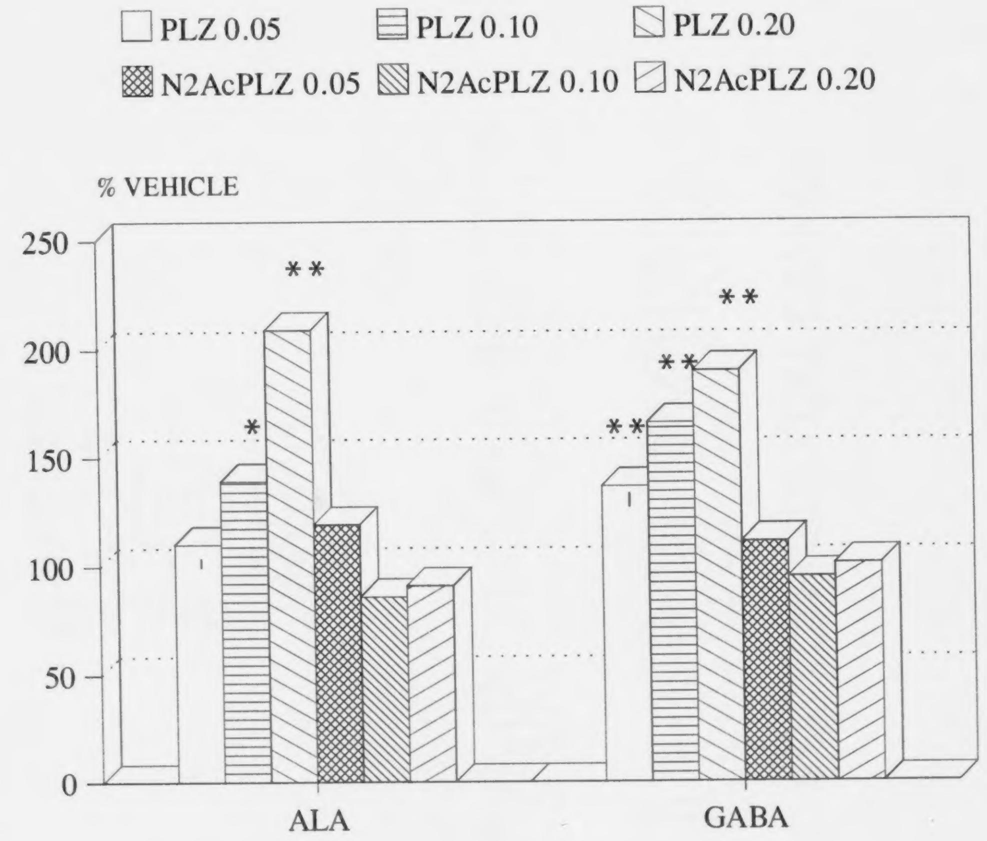

Figure 13: Effects of PLZ and N^{2}AcPLZ (0.05, 0.1 and 0.2 mmol/kg i.p.) on whole brain levels of GABA and ALA at 1 h post injection. Results expressed as % control, ± SEM, n=6. Control levels of GABA = 249 ± 6.8 μg/g; ALA = 60.3 ± 2.7 μg/g; n=18. * P<0.05; ** P<0.01, values compared to controls. The line in the clear box represents the standard error derived from the MSerror term.

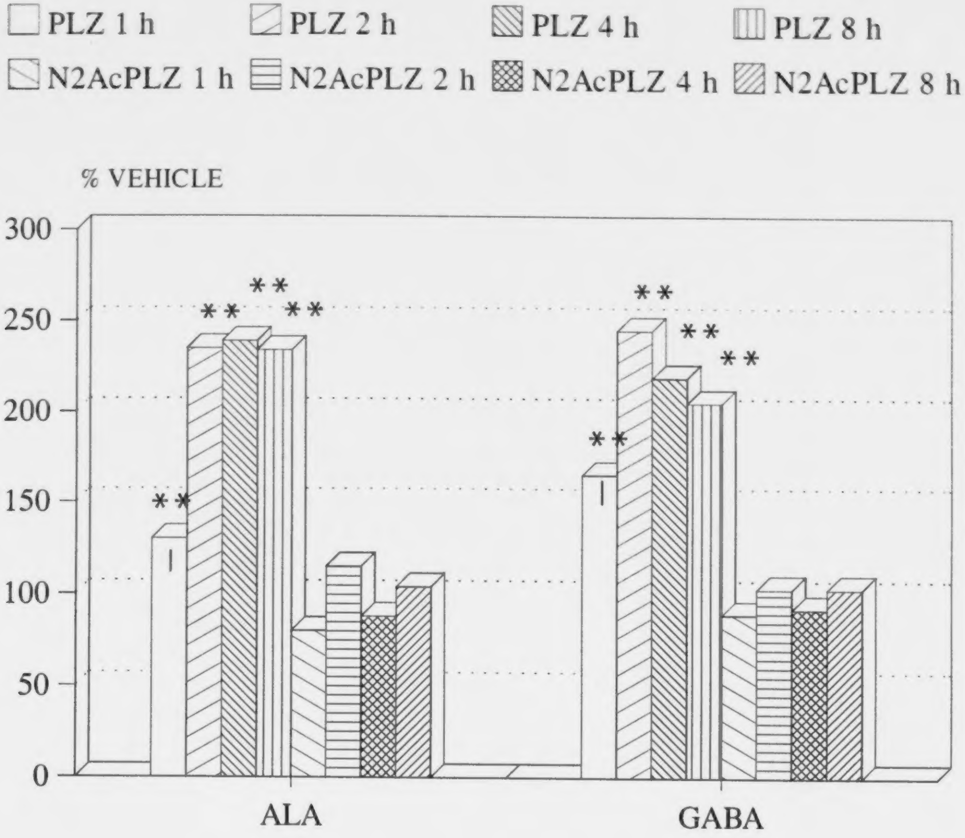

Figure 14: Effects of PLZ and N^{2}AcPLZ (0.1 mmol/kg i.p.) on whole brain levels of GABA and ALA at 1, 2, 4 and 8 h post injection. Results expressed as % control, ± SEM, n=6. Control levels of GABA = 274 ± 8.9 μg/g; ALA = 58.1 ± 3.2 μg/g; n=23. * $P<0.05$; ** $P<0.01$, values compared to controls. The line in the clear box represents the standard error derived from the MSerror term.

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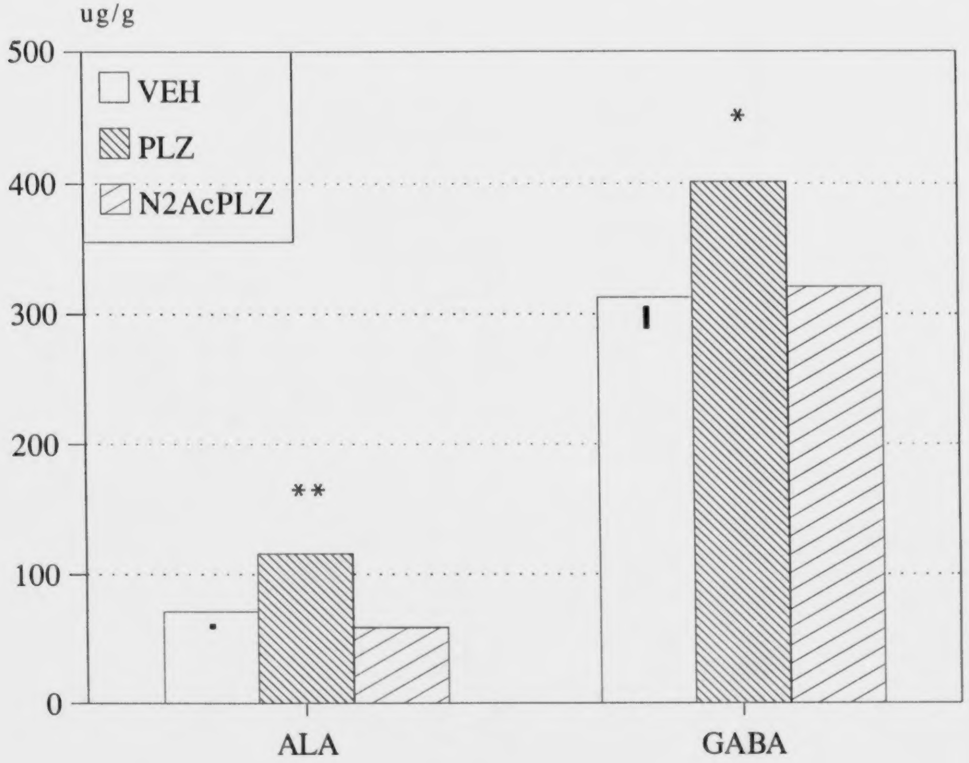

Figure 15: Effects of 28 d administration of PLZ (0.05 mmol/kg/d) and N^{2}AcPLZ (0.1 mmol/kg/d) on whole brain levels of GABA and ALA. Results expressed as mean concentration (μg/g), n=6. * P<0.05; ** P<0.01, values compared to controls. The line in the clear box represents the standard error derived from the MSerror term.

A.3.3 Effects of Pretreatment with MAO Inhibitors

In order to study the role of MAO in the elevation of GABA and ALA induced by PLZ, animals were pretreated with the MAOIs tranylcypromine (0.15 mmol/kg ip) and pargyline (0.38 mmol/kg ip) 15 min prior to administration of PLZ (0.11 mmol/kg) or VEH. Four hours following the second injection, animals were sacrificed and whole brains rapidly removed. The doses of TCP and PARG chosen were high enough to cause almost complete inhibition of MAO-A and -B activity. Whole brain levels of GABA and ALA for the 6 treatment groups (VEH/VEH, VEH/PLZ, TCP/VEH, TCP/PLZ, PARG/VEH, PARG/PLZ) are shown in Table 15. Significant elevations in GABA [F(5,41)=29.1; $P<0.01$] and ALA [F(5,41)=16.2; $P<0.01$] were seen only in the VEH/PLZ group; blockade of MAO-A and -B with TCP or PARG completely blocked the effect of PLZ, while having no effect on amino acids levels on their own.

A.4 Effects on GAD, GABA-T and ALA-T

The effects of chronic (28 d) treatment with PLZ (0.05 mmol/kg) and N^2AcPLZ (0.10 mmol/kg) on whole brain activity of the enzymes GAD, GABA-T and ALA-T are shown in Table 16. Chronic PLZ treatment induced a significant decrease in the activity of GAD [F(2,15)=6.5; $P<0.05$], GABA-T [F(2,15)=16; $P<0.01$] and ALA-T [F(2,15)=4.9; $P<0.05$] compared to N^2AcPLZ- and vehicle-treated animals. The effects of PLZ on GABA-T (33% inhibition) were more pronounced than on GAD (18% inhibition). The net effect appears to be an elevation in levels of GABA (see A.3). PLZ also significantly affected ALA-T (11%

Table 15: Effects of pretreatment (15 min) with VEH, TCP (0.15 mmol/kg i.p.) or PARG (0.38 mmol/kg) on the ability of PLZ (0.11 mmol/kg i.p.) to elevate ALA and GABA brain levels at 4 h post-administration. Results expressed as mean concentration (μg/g) ± SEM. ** $P<0.01$ compared to control (VEH/VEH) levels.

	ALA	GABA	n
VEH/VEH	58.7 ± 1.8	321 ± 10	11
VEH/PLZ	166 ± 20.0**	889 ± 77**	12
TCP/VEH	57.6 ± 4.2	316 ± 15	6
PARG/VEH	54.2 ± 7.3	318 ± 47	6
TCP/PLZ	57.2 ± 5.0	320 ± 20	6
PARG/PLZ	54.2 ± 1.2	270 ± 13	6

Table 16: Effects of chronic (28 d) administration of PLZ (0.05 mmol/kg/d) and N^2AcPLZ (0.1 mmol/kg/d) on whole brain activity of GAD, GABA-T and ALA-T (μmol/g/h ± SEM), n=6. * $P<0.05$ compared to VEH and N^2AcPLZ. ** $P<0.01$ compared to VEH and N^2AcPLZ. There were no significant differences between VEH and N^2AcPLZ.

	VEH	PLZ	N^2AcPLZ
GAD	10.59 ± 0.79	8.73 ± 0.21*	11.67 ± 0.59
GABA-T	28.25 ± 1.53	19.04 ± 0.98**	25.41 ± 0.94
ALA-T	2.47 ± 0.07	2.21 ± 0.08*	2.51 ± 0.07

inhibition) but this effect was less dramatic than in the case of GABA-T and GAD. There were no significant effects of N^2AcPLZ on GAD, GABA-T or ALA-T activity.

A.5 Effects on α_2-Adrenoreceptor Function

Both PLZ and N^2AcPLZ attenuated the motor suppressant effects of clonidine on days 21 and 22, suggesting a decreased α_2-receptor sensitivity (Figure 16). Median baseline activity counts in the PLZ and N^2AcPLZ groups (2132 and 2218 respectively) were significantly lower than those of the vehicle group (3332) [$P<0.05$]. It is possible that this baseline difference may have affected the apparent degree of attenuation of the clonidine response. Nevertheless, animals in the vehicle group exhibited lower activity counts following clonidine than the drug-treated animals, indicating the lack of a floor effect.

A.6 Effects on [^{3}H]-Muscimol Binding

The effects of chronic (28 d) administration of PLZ, N^2AcPLZ or vehicle (saline) on $GABA_A$ high-affinity receptor binding parameters (K_d and B_{max}) in rat brain cortex were studied using the $GABA_A$ agonist [^{3}H]-muscimol (Methods E.14). A competition assay was utilized in which a fixed concentration of [^{3}H]-muscimol (5 nM) was mixed with one of six concentrations of unlabelled muscimol (2-64 nM). Nonspecific binding was defined using GABA (1 mM). An analysis of the $GABA_A$ low-affinity binding site was unsuccessful using this assay, probably because of the rapid dissociation of [^{3}H]-muscimol from the binding site during the filtration and

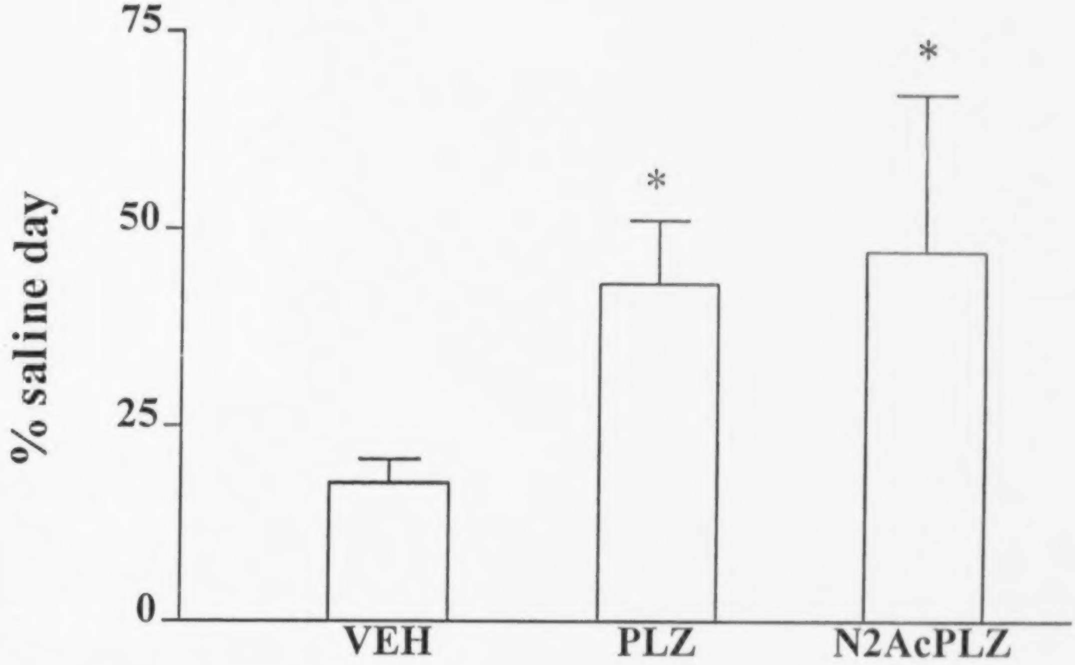

Figure 16: The effects of 21 d administration of PLZ (0.05 mmol/kg/d) and N^2AcPLZ (0.1 mmol/kg/d) on the degree of clonidine (50 μg/kg)-induced suppression of locomotor activity. The results are expressed as % of activity exhibited on the saline control day (clonidine activity/NaCl activity x 100%), n=10. The motor suppressant effect of clonidine was attenuated by chronic exposure to PLZ and N^2AcPLZ (* $P<0.05$).

Table 17: Effects of chronic (28 d) administration of PLZ (0.05 mmol/kg/d) and N^2AcPLZ (0.1 mmol/kg/d) on [^{3}H]-muscimol binding to rat brain cortex. Results expressed as mean ± SEM, n=6. There were no significant effects of treatment on the K_d or B_{max}.

	K_d (nM)	B_{max} (fmol/mg protein)
VEH	12.9 ± 0.9	834 ± 77
PLZ	12.1 ± 0.8	792 ± 51
N^2AcPLZ	12.2 ± 0.7	831 ± 35

washing steps.

The results are shown in Table 17. There were no significant differences in K_d or B_{max} between the treatment groups. The binding parameters are similiar to those reported by other investigators (Ito *et al.*, 1988; Kimber *et al.*, 1987; McManus, 1992).

A.7 Effects on [^{3}H]-Flunitrazepam Binding

The effects of chronic (28 d) administration of PLZ, N^2AcPLZ and vehicle (saline) on benzodiazepine receptor binding parameters (K_d and B_{max}) in rat brain cortex and hippocampus were studied using the benzodiazepine agonist [^{3}H]-flunitrazepam as a radioligand (Methods E.15).

The results are shown in Tables 18 and 19. There were no significant differences between the treatment groups on K_d or B_{max}. The addition of GABA to the homogenates increased the affinity of the benzodiazepine receptor (lower K_d) for [^{3}H]-flunitrazepam and increased the apparent density of binding sites (increase in B_{max}). This is in keeping with the hypothesis that GABA allosterically modulates the benzodiazepine binding site (Martin and Candy, 1978).

B. STUDIES OF HYDROXYLATION AS A PATHWAY OF PLZ METABOLISM

B.1 Effects of Iprindole on PLZ and β-Phenylethylamine Levels

In order to investigate the possibility of ring hydroxylation as a metabolic route for PLZ in the rat, iprindole, a drug known to block ring hyroxylation (Freeman

Table 18: Effects of chronic (28 d) administration of PLZ (0.05 mmol/kg/d) and N^2AcPLZ (0.10 mmol/kg/d) on ^{3}H-flunitrazepam binding to membrane fractions from rat brain cortex. Results expressed as mean ± SEM (n=6). The two columns on the right demonstrate the effects of adding GABA to the incubation mixture. There were no significant effects of drug treatment on K_d or B_{max}.

			+ GABA (10^{-4} M)	
	K_d (nM)	B_{max} (fmol/mg protein)	K_d (nM)	B_{max} (fmol/mg protein)
VEH	1.32 ± 0.08	971 ± 48	0.83 ± 0.04	1,108 ± 32
PLZ	1.45 ± 0.05	1,015 ± 29	0.85 ± 0.04	1,238 ± 36
N^2AcPLZ	1.44 ± 0.10	986 ± 36	0.80 ± 0.04	1,165 ± 35

Table 19: Effects of chronic (28 d) administration of PLZ (0.05 mmol/kg/d) and N^2AcPLZ (0.10 mmol/kg/d) on ^{3}H-flunitrazepam binding to membrane fractions from rat brain hippocampus. Results expressed as mean ± SEM (n=6). The two columns on the right demonstrate the effects of adding GABA to the incubation mixture. There were no significant effects of drug treatment on K_d or B_{max}.

			+ GABA (10^{-4} M)	
	K_d (nM)	B_{max} (fmol/mg protein)	K_d (nM)	B_{max} (fmol/mg protein)
VEH	1.32 ± 0.05	795 ± 47	0.48 ± 0.02	969 ± 50
PLZ	1.39 ± 0.08	826 ± 63	0.54 ± 0.02	1,039 ± 81
N^2AcPLZ	1.35 ± 0.11	812 ± 65	0.49 ± 0.07	985 ± 50

and Sulser, 1972; Callaghan *et al.*, 1985) was administered to rats followed by PLZ injection. Rats were administered intraperitoneal (i.p.) injections of vehicle or of iprindole HCl (11.2 mg/kg) dissolved in 0.9% NaCl. One h later rats were injected i.p. with vehicle or with PLZ [sulfate salt] (0.2 mmol/kg) dissolved in 0.01 M sodium phosphate buffer (pH=7.4). Animals were sacrificed by decapitation at 1 and 3 h after this second injection and whole brains were immediately removed and frozen solid in isopentane on solid $C0_2$. The effects of treatment on brain levels of PLZ and PEA are shown in Table 20. Whole brain levels of PLZ are significantly higher at 1 h after administration than at 3 h in the VEH/PLZ treated groups. Pretreatment with iprindole results in significantly higher PLZ and PEA levels compared to controls (VEH/PLZ) at 3 h after PLZ administration. In the presence of iprindole, PLZ levels at 3 h were similiar to the levels normally seen at 1 h after administration. PEA levels also rose at 3 h, presumably due in part to metabolism of PLZ to PEA. These results show that PLZ levels in rat brain are raised following pretreatment with a drug that blocks ring hydroxylation. These observations provide indirect evidence that PLZ is ring-hydroxylated to *p*-OHPLZ.

Unequivocal evidence of ring hydroxylation would require identification of *p*-OH-PLZ as a metabolite following PLZ administration. In order to try and provide such evidence, I attempted to develop an assay procedure for *p*-OH-PLZ and apply it to urine samples collected from patients treated with PLZ. Attempts to use acetylation under aqueous conditions (Martin and Baker, 1977) or to derivatize with pentafluorobenzenesulfonyl chloride or pentafluorobenzoyl chloride under aqueous conditions (Rao *et al.*, 1986, 1987) followed by gas chromato-graphic analyses were not successful. A procedure developed in the Neurochemical Research Unit for

Table 20: Effects of pretreatment with IPR (1 h previous) on rat whole brain levels of PLZ and PEA. Concentrations of PLZ and PEA are expressed as ng/g (mean ± SEM), n=8. * $P<0.05$ compared to values in treatment group (C).

DRUG TREATMENT	PLZ	PEA
(A) VEH/PLZ, 1 h	183 ± 11	29 ± 5
(B) IPR/PLZ, 1 h	209 ± 31	27 ± 4
(C) VEH/PLZ, 3 h	109 ± 11	28 ± 4
(D) IPR/PLZ, 3 h	205 ± 34*	46 ± 6*

analysis of *p*-tyramine (Coutts *et al.*, 1980) proved to be useful for derivatization of *p*-OH-PLZ as well. The proposed reaction sequence for the derivatization is shown in Figure 17. Derivatization of the extracted urine samples resulted in a sharp peak (Figure 18) which had a retention time on several columns identical to that of derivatized authentic *p*-OH-PLZ. Unfortunately, attempts to unequivocally establish the structure of the derivative by combined GG/MS were unsuccessful. No molecular ion was apparent, and the proposed mass fragmentation pathway, although highly suggestive of the structure shown in Figure 19, did not provide unequivocal proof of the structure.

B.2 *p*-OH-PLZ in Humans and Rats Taking PLZ

The urinary concentrations of *p*-OH-PLZ in humans administered PLZ for the treatment of psychiatric disorders are shown in Table 21. These analyses were made on the assumption that the peak in the urine samples with the same retention time as authentic *p*-OH-PLZ was in fact *p*-OH-PLZ. As mentioned in section B.1 above, I have not be able to unequivocally identify *p*-OH-PLZ in the samples using GC/MS. Another problem encountered was that the conjugated form of the *p*-OH-PLZ appears to be unstable. When urine samples were sampled weekly over 6 weeks, the levels of conjugated *p*-OH-PLZ decreased progressively. Therefore the results given here should be considered qualitative or semiquantitative rather than quantitative.

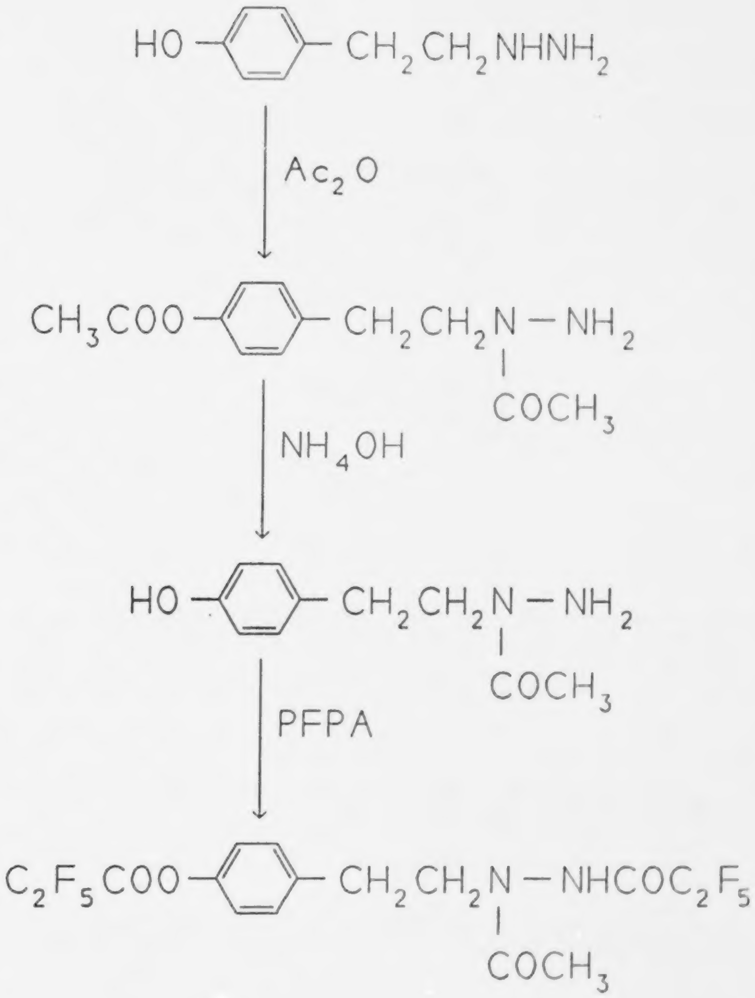
HO
CH2CH2NHNH2
Ac2O
CH3COO
CH2CH2N − NH2
COCH3
NH4OH
HO
CH2CH2N − NH2
COCH3
PFPA
C2F5COO
CH2CH2N − NHCOC2F5
COCH3

Figure 17: Reaction sequence for the derivatization of *p*-OH-PLZ.

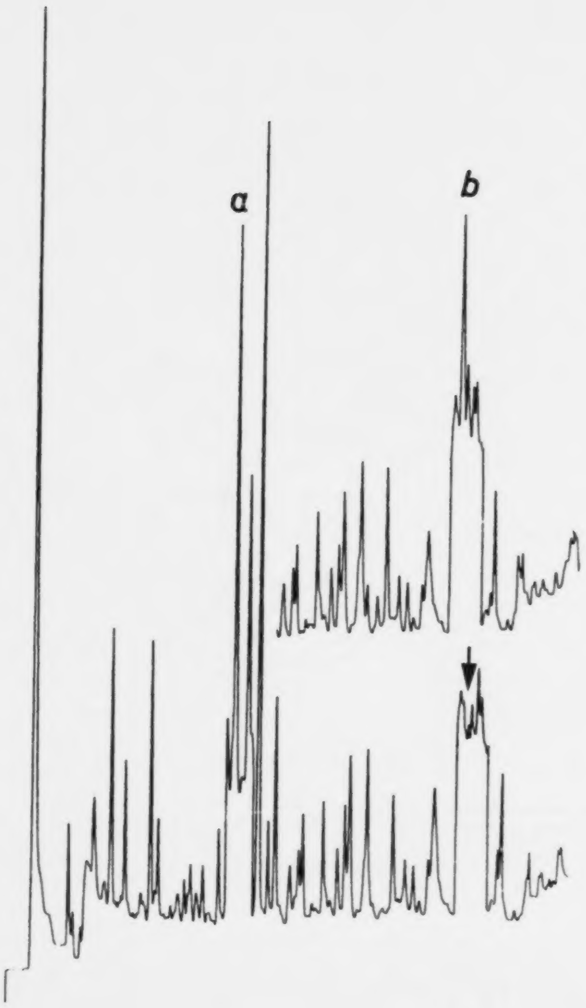

Figure 18: Typical gas chromatographs obtained from extracts of human urine samples derivatized as described in section E.6. The bottom trace is a derivatized extract of a urine sample for a patient pretreatment; the top trace is a derivatized extract of a urine sample from the same patient after treatment with PLZ for 2 weeks. a=derivatized added internal standard (benzylamine); b=derivatized *p*-OH-PLZ.

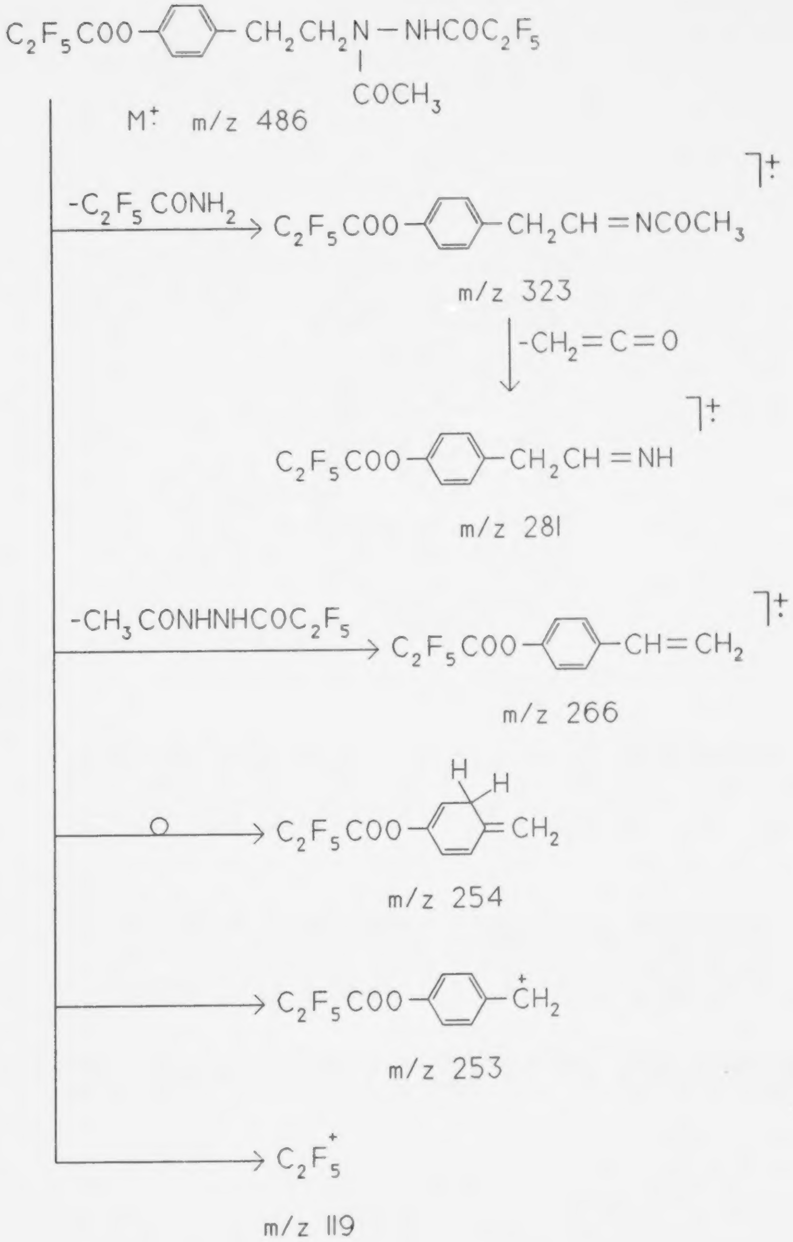
C2F5COO-C6H4-CH2CH2N(COCH3)-NHCOC2F5
M+· m/z 486
-C2F5CONH2
C2F5COO-C6H4-CH2CH=NCOCH3]+·
m/z 323
-CH2=C=O
C2F5COO-C6H4-CH2CH=NH]+·
m/z 281
-CH3CONHNHCOC2F5
C2F5COO-C6H4-CH=CH2]+·
m/z 266
C2F5COO-C6H4(H)(H)=CH2
m/z 254
C2F5COO-C6H4-CH2+
m/z 253
C2F5+
m/z 119

Figure 19: Proposed mass fragmentation for the derivative of *p*-OH-PLZ.

Table 21: Urinary concentrations of *p*-OH-PLZ in urine samples collected from patients taking PLZ for 2 weeks and 4 weeks. Results are expressed as μg/g creatinine (mean ± SEM, n=7). ND=not detected.

Metabolite	Pretreatment	2 weeks	4 weeks
p-OH-PLZ (free)	N.D.	38 ± 8	45 ± 14
p-OH-PLZ (total=free + conjugated)	N.D.	277 ± 100	356 ± 84

B.3 Effects of *p*-OH-PLZ on Neurotransmitter Uptake and Release

PLZ can inhibit the uptake of, and stimulate the release of, catecholamines from nerve-ending fractions prepared from brain (Hendley and Snyder, 1968; Baker *et al.*, 1978, 1980; Dyck, 1983). In general, the addition of a *p*-hydroxy group to the aromatic ring of a phenylethylamine nucleus results in increased ability to affect the uptake and release of catecholamines and 5-HT (Raiteri *et al.*, 1977), so the effects of PLZ and *p*-OH-PLZ on the uptake and release in slices prepared from striatum (DA, 5-HT) or hypothalamus (NA) were compared. The results of the uptake and release studies are shown in Tables 22 and 23. *p*-OH-PLZ was slightly more potent than PLZ at stimulating release of [^{3}H]-NA from hypothalamic slices and markedly stronger at stimulating release of [^{3}H]-DA and [^{3}H]-5-HT from striatal slices (Table 22). *p*-TA was included in the studies as a classical releaser of all three biogenic amines (Raiteri *et al.*, 1977) and was shown to be more potent than *p*-OH-PLZ at stimulating release of all three neurotransmitter amines. In the uptake studies (Table 23), *p*-OH-PLZ was more potent than PLZ at inhibiting uptake of all three neurotransmitter amines.

C. EFFECTS OF PLZ IN HUMAN SUBJECTS

C.1 Clinical Effects

Of the 13 subjects, 8 noticed a marked improvement in symptomatology. The subjects that responded had a mixture of anxiety (panic, agoraphobia, generalized anxiety and social phobia) and affective (major depression, dysthymia and bipolar) disorders (see Table 24).

Table 22: Effects of PLZ, *p*-OH-PLZ and *p*-TA on release of ^{3}H-neurotransmitter amines from striatal or hypothalamic slices. Values represent % increase over control values and are expressed as mean ± SEM. Drug concentrations were 10^{-5}M in all cases. Superscripts: significantly different ($P<0.05$) from: control[a]; PLZ[b]; *p*-OH-PLZ[c]; and *p*-TA[d].

	N	^{3}H-DA release (striatum)	^{3}H-NA release (hypothalamus)	^{3}H-5-HT release (striatum)
PLZ	6	$112.6^{a,c,d} \pm 3.3$	$115.7^{c,d} \pm 8.6$	$107.7^{c,d} \pm 3.0$
p-OH-PLZ	6	$156.3^{a,b,d} \pm 8.1$	$126.9^{a,b,d} \pm 9.5$	$141.1^{a,b,d} \pm 7.9$
p-TA	3	$324.4^{a,b,c} \pm 12.8$	$158.6^{a,b,c} \pm 13.9$	$250.6^{a,b,c} \pm 11.4$

Table 23: Effects of PLZ and *p*-OH-PLZ on uptake of ^{3}H-neurotransmitter amines into striatal or hypothalamic slices. Values represent mean IC$_{50}$ values (μM) ± SEM (n=6).

	^{3}H-DA (striatum)	^{3}H-NA (hypothalamus)	^{3}H-5-HT (striatum)
PLZ	18.9 ± 7.8	6.6 ± 1.2	155.0 ± 26.8
p-OH-PLZ	6.8 ± 0.4	2.6 ± 0.2	6.1 ± 1.2

Table 24: Characteristics of the human subjects taking PLZ in this study. [a]MDD (Major Depressive Disorder), MDD +P (MDD + Psychoses), BAD (Bipolar Affective Disorder), PD (Panic Disorder), PD + A (PD + Agoraphobia), GAD (Generalized Anxiety Disorder), SP (Social Phobia), D (Dysthymia), AA (Alcohol Abuse), BPD (Borderline Personality Disorder).

[b]CGI-I (Clinical Global Impression-Improvement); 1=Very Much Improved, 2=Much Improved, 3=Minimally Improved, 4=No Change, 5=Minimally Worse, 6=Much Worse, 7=Very Much Worse.

	Diagnoses[a]	Sex	Age	Dose (mg/d)	CGI-I[b]
1	MDD + P,AA	M	45	90	4
2	MDD	F	20	45	1
3	D,PD + A,AA,BPD	F	30	60	2
4	PD + A	F	32	45	3
5	BAD,SP,PD + A	M	31	60	1
6	D,PD,GAD	F	31	30	4
7	SP	M	27	30	4
8	BAD,PD,BPD	F	33	45	2
9	MDD,PD + A	F	35	45	5
10	D,SP,AA	M	20	45	2
11	MDD,GAD	F	51	45	1
12	MDD,PD + A, GAD	F	31	45	1
13	MDD,SP,PD + A, GAD	F	27	45	1
	MEDIAN (SIQ)	4M,9F	31 (27-32)	45 (45-45)	2 (1-4)

C.2 Effects on 5-HT and Trace Amines

The effects of chronic PLZ administration on urinary excretion of 5-HT and the trace amines (T, TA and PEA) are shown in Table 25. The urinary levels of PEA, *m*- and *p*-TA, T and 5-HT were dramatically increased following PLZ treatment. The effects of PLZ were most marked on the excretion of PEA and T of the amines studied.

C.3 Effects on Metabolites of the Classical Neurotransmitters and Trace Amines

The effects of chronic PLZ treatment on urinary excretion of the metabolites of the classical neurotransmitters (5-HIAA, HVA, MHPG, 3-MT and NME) and trace amines (PAA, *m*- and *p*-OH-PAA) are shown in Table 26. Urinary levels of the NA metabolites NME and MHPG are increased and decreased respectively during treatment with PLZ; a similar pattern was observed with the DA metabolites 3-MT and HVA. Such effects would be expected from a drug that inhibits MAO. The 5-HT metabolite 5-HIAA was not significantly decreased in the present study. There was an elevation in levels of *m*-TA and a decrease in its acid metabolite *m*-OH-PAA. No significant changes were noted in the levels of PAA and *p*-OH-PAA.

Table 25: Effects of administration of PLZ on urinary excretion of trace amines and 5-HT in psychiatric patients. Results expressed as mean (SEM), μg/g creatinine, n=13 and represent free (unconjugated) values. *P<0.05; **P<0.01, significantly different from pretreatment values.

	Pretreatment	**2 Weeks**	**4 Weeks**
PEA	6 (1)	81 (26)**	108 (31)**
p-TA	307 (33)	848 (82)**	1136 (236)**
m-TA	76 (14)	178 (21)**	232 (42)**
T	84 (10)	730 (112)**	778 (130)**
5-HT	79 (5)	147 (14)*	217 (44)**

Table 26: Effects of administration of phenelzine on urinary excretion of metabolites of trace amines and of classical neurotransmitter amines in psychiatric patients. Results are expressed as mean (SEM), μg/g creatinine (except for PAA, where the units are mg/g creatinine), n=13. Levels for MHPG and PAA are total (free + conjugated) values; all others represent free (unconjugated) values. *P<0.05; **P<0.01, significantly different from pretreatment values.

	Pretreatment	**2 Weeks**	**4 Weeks**
5-HIAA	4767 (642)	3282 (576)	3659 (771)
HVA	3286 (418)	1740 (309)**	1256 (271)**
MHPG	2476 (296)	1547 (229)**	808 (224)**
3MT	71 (32)	120 (36)**	190 (51)**
NME	48 (9)	107 (15)**	143 (22)**
PAA	74 (7)	100 (25)	118 (26)
m-OHPAA	5807 (1343)	3718 (807)	1574 (593)**
p-OHPAA	24682 (6125)	20035 (4708)	22767 (7643)

DISCUSSION

A. NEUROCHEMICAL EFFECTS OF PLZ AND N^2AcPLZ IN RATS

A.1 Inhibition of MAO

Both PLZ and N^2AcPLZ are potent inhibitors of MAO-A and -B. PLZ is more potent than N^2AcPLZ following acute administration and *in vitro*, but this difference was not evident following 28 days of administration of the drugs at the doses used in this study (Table10). Both drugs are slightly more potent at inhibiting MAO-A than MAO-B *ex vivo* and *in vitro* after acute exposure. Following chronic administration at the doses used in this study, no difference was seen in the selectivity of either PLZ or N^2AcPLZ.

It may be hypothesized that N^2AcPLZ would have many of the neurochemical effects common to the MAOIs and may have beneficial clinical effects on the basis of its ability to nonselectively inhibit MAO. This study includes the first description of the chronic effects of N^2AcPLZ on MAO activity. Previous preliminary studies in this laboratory had established that it was an MAOI *in vitro* and *ex vivo* following acute administration in rats (Danielson *et al.*, 1984, 1988), but the studies were much less comprehensive than those described here.

A.2 Biogenic Amines and Metabolites

As a consequence of nonselective MAO inhibition, it is to be expected that brain levels of the neurotransmitter amines (NA, DA and 5-HT) would increase and their acid metabolites (DOPAC, HVA, 5-HIAA) would decrease. This pattern was observed following both acute and chronic administration of PLZ and N^2AcPLZ in rats. A higher dose of N^2AcPLZ was necessary to significantly increase whole brain levels of NA, DA (0.20 mmol/kg) and 5-HT(0.10 mmol/kg) at 1 h, but in the time course study the effects of PLZ and N^2AcPLZ were quite comparable. The dose-response findings are similiar to those completed independently by Coutts *et al.* (1991). In the time-course study, at 8 h post-administration, the longest time interval studied acutely, there were no significant differences between the active treatments. N^2AcPLZ may have a longer time-course of effect but the inclusion of further time-intervals (e.g. 12 and 24 h) would be necessary to demonstrate this. The increasing inhibition of MAO with time in the case of N^2AcPLZ is interesting and suggests that N^2AcPLZ may be highly concentrated in brain and increasing amounts are metabolized to PLZ with time. However, Coutts *et al.* (1991) did not observe such a pattern in rat blood or liver.

Following chronic (28 d) administration of PLZ and N^2AcPLZ with continuous subcutaneous infusion via osmotic minipumps, sustained elevations in brain catecholamine and 5-HT levels were observed. These results are different from those of some previous studies of chronic PLZ administration that suggested the elevations in NA, 5-HT and DA were transient (Campbell *et al.*, 1977; Robinson *et al.*, 1979), but in agreement with other reports in the literature (Baker *et al.*, 1984a,b; Blier *et al.*, 1986; Juorio *et al.*, 1986). Sustained increases that are most pronounced for 5-HT (3-fold) and NA (2-fold) were observed following 28 days of treatment. By this time, each drug had inhibited MAO-A and -B by >90%. Consistent with the results of the acute studies, administration of PLZ resulted in a greater decrease in 5-HIAA levels than did N^2AcPLZ following 28 d of treatment. In this regard, it is of interest that chronic administration of PLZ has also been reported to produce a larger decrease in urinary levels of 5-HIAA in rats than does brofaromine, even though both drugs inhibited MAO-A, the isozyme that preferentially deaminates 5-HT, to a similar extent (Urichuk *et al.*, 1994). The levels of the acid metabolites of DA, namely DOPAC and HVA, were decreased so markedly that they were routinely beneath the threshold of detection (about 10 ng/g) in both groups following 28 d of drug administration.

A.3 GABA and Alanine

The most notable differences between PLZ and N^2AcPLZ were observed in their effects on levels of the amino acids GABA and ALA and on the activity of GAD, GABA-T and ALA-T. PLZ had dramatic effects, elevating brain levels of both GABA and ALA following both acute and chronic administration. In marked contrast to PLZ, N^2AcPLZ had no effects on brain levels of GABA or ALA. Even at a dose of 0.50 mmol/kg, no effect of N^2AcPLZ on brain levels of GABA or ALA was observed. In the acute studies, the maximal effect of PLZ on GABA and ALA levels was seen at 2 and 4 h post-dose respectively, but the effect was still pronounced at the 8 h interval. In studies completed previously by Wong *et al.* (1990b) and Baker *et al.* (1991), the minimal dose of PLZ necessary to increase brain levels of GABA and ALA (at 4 h post-dose i.p.) were 0.02 mmol/kg and 0.03 mmol/kg respectively. These workers found that the maximal effect was observed at 4 h following a single i.p. dose and the levels gradually declined over the ensuing 24 h. These findings are in agreement with those of the present study.

The chronic study reported in this thesis represents the first comparison of chronic effects of PLZ and N^2AcPLZ on aliphatic amino acid levels. As in the acute studies, PLZ caused a significant increase in brain GABA and ALA levels, while results with N^2AcPLZ were not significantly different from control animals. Following chronic administration of PLZ, GABA and ALA levels were elevated to 128% and 163% of control levels respectively. Thus, the effects of PLZ on GABA and ALA are sustained, and the magnitude of increase in ALA levels seemed to be more pronounced than that in GABA levels.

By modifying the hydrazine group of PLZ with the addition of an acetyl moiety, a drug (N^2AcPLZ) has been produced that lacks effects on GABA and ALA concentrations. Elevations of brain GABA and ALA concentrations by acute administration of PLZ have been reported by others (Popov and Matthies, 1969; Perry and Hansen, 1973; Baker *et al.,* 1991; Wong *et al.,* 1990b), and these increases may be due, at least in part, to inhibition of transaminases for these enzymes (Popov and Matthies, 1969; Baker and Martin, 1989). The hypothesis that inhibition of the transaminase enzymes GABA-T and ALA-T is an important mechanism through which PLZ elevates GABA and ALA levels was tested by examining the effects of PLZ on activity of these enzymes. Following chronic administration (28 d) of PLZ, a significant inhibition of the activities of GAD, GABA-T and ALA-T were observed (Table 19). This was greater for GABA-T (33% inhibition) than for ALA-T (11% inhibition) and GAD (18% inhibition). The GAD results seem paradoxical as inhibition of GAD should result in lower concentrations of GABA. The observed net effect of the GAD and GABA-T inhibition is an increase in GABA levels, indicating that the inhibition of GABA-T is the predominant effect. As both GAD and GABA-T are pyridoxal-5'-phosphate-dependent (Dakshinamurti *et al.,* 1992), an inhibition of this cofactor would explain the simultaneous inhibition of the anabolic and catabolic GABA enzymes. In this regard, it is of interest that Yu *et al.* (1994) have recently reported a reduction in plasma pyridoxal phosphate levels in patients who have been treated with PLZ. Lydiard *et al.* (1989) did not find a change in plasma levels of pyridoxal phosphate in PD patients treated with PLZ, but indicated that some of the patients in their study took vitamin supplements during the course of the investigation [in the study by Malcolm *et al.*, (1994) the patients were not taking vitamin supplements].

Previous experiments have documented the inhibition of ALA-T (Baker and Martin, 1989) and GABA-T (Popov and Matthies, 1969; Baker and Martin, 1989) following acute treatment of rats with PLZ. GABA is catabolized in the mitochondria of neurones and in glial cells by the combined action of GABA-T and succinic semialdehyde dehydrogenase which require pyridoxal 5'-phosphate as a cofactor and NAD^+ as a coenzyme (McGeer and McGeer, 1989). Phenelzine probably increases levels of GABA and ALA by the interaction of the hydrazine group of PLZ with pyridoxal 5'-phosphate, an effect observed in the presence of several hydrazine drugs (review; Tunnicliff, 1989) and apparently blocked by acetylation in the N^2-position of PLZ. It is possible that N-acetylation of the hydrazine group will produce a compound with fewer side-effects than the parent drug. Side-effects reported with PLZ include pyridoxine deficiency (Robinson and Kurtz, 1987; Stewart *et al.*, 1984; Malcolm *et al.*, 1994) and a lupus-like reaction (Swartz, 1978; Uetrecht, 1988). In this regard, it is interesting that the antiarrythmmic drug procainamide is known to precipitate lupus erythematosus, while N-acetylprocainamide does not produce this toxic action (Uetrecht, 1988).

The elevations of GABA and ALA following administration of PLZ do not appear to be simply attributable to a direct inhibition of transaminase enzymes for these amino acids by PLZ. In the original description of the effects of PLZ on GABA levels (Popov and Matthies, 1969), it was noted that pretreatment with TCP blocked the effects of PLZ on GABA, thus suggesting that MAO activity was necessary for the PLZ-induced elevation of GABA. An *ex vivo* experiment was conducted in the present study to verify and extend these preliminary findings. The MAOI pargyline (a propargylamine) was included to ascertain that this blocking action of the GABA-elevating effects of PLZ was not restricted to TCP (a phenylcyclopropylamine). Pretreatment of rats with the MAOIs TCP and pargyline (nonselective inhibitors of MAO-A and -B at the doses employed) completely blocked the elevation of GABA and ALA induced by PLZ (Table 20). These findings support the original work by Popov and Mathies and indicate that intact MAO is necessary for PLZ to raise GABA and ALA levels. This observation is most easily explained by the formation of an active metabolite of PLZ produced by the interaction of PLZ with MAO. This metabolite may then produce the elevation of GABA and ALA levels. Further work is now necessary to elucidate the metabolite involved, but a reasonable hypothesis is that a hydrazine-containing compound, perhaps even hydrazine, is responsible. Hydrazine has been reported previously to inhibit GABA-T and elevate GABA (Perry *et al.*, 1981). A hydrazine-containing metabolite with GABA-elevating properties would explain why N^2AcPLZ, which presumably would not be readily metabolized to hydrazine or to a metabolite containing a free hydrazine group has no effect on the amino acids studied.

It is well known that PLZ is a substrate for, as well as an inhibitor of, MAO

(Clineschmidt and Horita, 1969a,b; Tipton and Spires, 1971; Strolin Benedetti and Dostert, 1994), but the nature of the metabolite(s) responsible for the GABA-elevating effect of PLZ is still not known. Phenylacetic acid and *p*-hydroxyphenylacetic acid are identified metabolites of PLZ (Clineschmidt and Horita, 1969b; Robinson *et al.*, 1985), but neither of these compounds inhibits GABA-T *in vitro* (Baker, Leung and McKenna, unpublished). Tipton and Spires (1971) and Patek and Hellerman (1974) suggested that phenylethylidene hydrazine or phenylethyldiazene, respectively, may be formed, but this has not been confirmed by other workers, nor are the effects of these possible metabolites on GABA known. *p*-OH-PLZ has some GABA-T-inhibiting properties *in vitro* (Baker, McKenna and Coutts, unpublished), but it is unlikely that formation of this metabolite would be decreased by prior pretreatment with MAOIs. It is also of interest that Todd *et al.* (1995) recently tested both (-)-deprenyl and clorgyline, at doses at which they were selective for inhibition of MAO-A and -B, respectively, and found that (-)-deprenyl was more potent than clorgyline at blocking the GABA-elevating effect of PLZ. This finding suggests that MAO-B may be more important than MAO-A at mediating the formation of the GABA-elevating metabolite from PLZ.

Since inhibition of both GABA-T and ALA-T by PLZ is relatively low, it is also possible that inhibition of these enzymes may not be the sole factor responsible for the elevations of GABA and ALA levels in brain. Recent studies by Paslawski *et al.* (1994) have shown that PLZ also produces a decrease in brain levels of glutamine. GABA is formed in the metabolic pathway referred to as the GABA shunt (McGeer and McGeer, 1989), and formation of glutamine is closely associated with the GABA shunt through another loop in which GABA is taken up by glial cells and metabolized by GABA-T. The glial cells lack GAD, so the glutamate formed in the transamination reaction is transformed by glutamine synthetase into glutamine which can be returned to the nerve ending to be converted back to glutamate by the enzyme glutaminase (McGeer and McGeer, 1989). It is thus feasible that PLZ may also be altering levels of GABA through effects on glutaminase or glutamine synthetase, but such actions have not yet, to my knowledge, been investigated.

It should also be considered that PLZ and/or its metabolite(s) may just be acting as general inhibitors of transaminase enzymes. It is of interest in this regard that Dyck and Dewar (1986) have also reported inhibition of transamination of tyrosine by PLZ. However, the amino acids leucine, isoleucine and valine are also metabolized by transaminases, and, as shown in this thesis, PLZ has no effect on their brain levels.

At present, there is a paucity of information available on the role of ALA in the central nervous system or its possible involvement in the etiology of depression or PD. ALA is present in various species at concentrations 25-60% of those of GABA. It has been reported that levels of this amino acid are increased in brain following administration of the convulsant pentamethylenetetrazole (Clarke *et al.*, 1989) and that, like glycine, ALA can activate NMDA excitatory amino acid receptors although it is weaker than glycine in this effect (Thomson, 1989).

Levels of ALA in rat cerebral cortex have been reported to be elevated during hypocapnia (Norberg, 1976), hypoxia (Norberg and Siesjo, 1975), seizures (Chapman *et al.*, 1977) and ischemia (Folbergrova *et al.*, 1974). Studies in cultured astrocytes (Yudkoff *et al.*, 1986, 1988) and cerebrocortical GABAergic neurons (Yudkoff *et al.*, 1990) suggest that ALA is a major repository of glutamate- and glutamine-nitrogen. In a recent comprehensive report, Erecinska *et al.* (1994) concluded that ALA increases release of glutamate from nerve endings and that ALA-T might serve as a source of glutamate during recovery from ischemia/hypoxia.

ALA is related metabolically, via transamination, to pyruvate and lactate, and the latter substance is known to produce panic attacks in certain individuals (Shear, 1986). It is interesting to consider the possibility that the increase in brain levels of ALA may reflect some decreased lactate formation and that this may contribute to the known antipanic effects of PLZ. ALA is also known to inhibit glutamine synthetase (Stryer, 1975), and thus the ALA accumulation in brain following administration of PLZ may account for the decreased glutamine levels in brain observed by Paslawski *et al.* (1994) after PLZ administration.

A series of radioligand binding experiments was conducted to determine if the sustained elevation in GABA levels was associated with changes in $GABA_A$ and benzodiazepine receptor binding parmeters (K_D and B_{max}). No significant differences between treatment groups were noticed in these experiments. The $GABA_A$ high affinity binding site in cortical tissue was studied initially. The protocol did not allow characterization of the low affinity binding site due to rapid dissociation of the ligand from the receptor during the washing steps. There were no significant differences between the treatment groups. The benzodiazepine receptor was studied in cortical and hippocampal tissue and the modulating effects of GABA were also examined. The investigation of the modulating effects of GABA on $[^3H]$-flunitrazepam binding was of interest as this would reflect the function of the low affinity $GABA_A$ binding site. It is generally thought that the lower affinity $GABA_A$ site is functionally linked to the $GABA_A$/benzodiazepine complex (Unnerstahl *et al.*, 1981). If there were no differences in the benzodiazepine binding parameters, but a difference appeared with the addition of GABA it would be suggestive that the $GABA_A$ low affinity site was being altered by the treatment. In both cortical and hippocampal tissue, there were no significant differences in benzodiazepine receptor parameters between the groups even after the addition of GABA. As previously reported, the addition of GABA and NaCl to the binding suspension resulted in a significant increase in affinity ($\downarrow K_D$) of the benzodiazepine receptor for [^{3}H]-flunitrazepam and a significant increase in the density of binding sites (B_{max}), but this was uniform between the treatments.

Despite the dramatic and sustained elevation in GABA levels, PLZ did not appear to significantly affect binding parameters of the $GABA_A$-benzodiazepine receptor complex as assayed with radioligand binding procedures. Other groups which have studied the effects of antidepressants on $GABA_A$ and benzodiazepine binding sites have reported contradictory results. Pilc and Lloyd (1984) failed to find an effect of antidepressants on $GABA_A$ binding sites but did not distinguish between low or high affinity sites. In contrast, Suzdak and Gianutsos (1985) observed that chronic administration of IMI or nomifensine induced a significant reduction in the density of high and low affinity $GABA_A$ sites in mouse cerebral cortex. Suranyi-Cadotte and colleagues (1984) described a decrease of benzodiazepine binding site density following treatment with DMI, buproprion, maprotiline, zimelidine and adinazolam, but Kimber *et al.* (1987) and Przegalinski *et al.* (1987) were unable to find any such effects after chronic administration of a variety of antidepressants. Todd *et al.* (1992) did not observe down-regulation of benzodiazepine receptors with several antidepressants (including PLZ), even at doses which induced marked down-regulation of 5-HT_2 receptors.

There is a relatively large (albeit controversial) body of evidence suggesting a possible role of GABA in the actions of antidepressant and antipanic drugs (Lloyd *et al.*, 1989; Breslow *et al.*, 1989), and it is feasible that the increase in GABA observed with PLZ may contribute to its action in both depression and PD. Lower than normal GABA levels have been found in both cerebrospinal fluid (Gold *et al.*, 1980; Gerner and Hare, 1981; Kasa *et al.*, 1982) and plasma (Berrettini *et al.*, 1982; Petty and Sherman, 1982; Petty *et al.*, 1993) of depressed patients. This reduction in GABA was not confirmed in a study by Korpi *et al.* (1988) in postmortem brain tissue from depressed suicide victims, but it should be remembered there are marked postmortem changes in GABA concentrations in brain and these may have masked any changes. Reduced activity of the GABA synthetic enzyme GAD in the frontal cortex from depressed patients (as compared to controls) was reported by Perry *et al.* (1977). Anticonvulsant drugs such as sodium valproate (Lambert *et al.*, 1975; Emrich *et al.*, 1980) and carbamazepine (Okuma *et al.*, 1973; Ballenger and Post, 1980), both of which have been used in the treatment of lithium-resistant affective disorders, induce a decrease in GABA turnover in rat brain (Bernasconi and Martin, 1979). Electroconvulsive shocks, comparable to electroconvulsive therapy used in the treatment of depression, alter the concentration and synthesis of GABA in rat nucleus accumbens and caudate nucleus when administered chronically (Green *et al.*, 1978). Antidepressant effects have been observed with a diverse range of GABA agonists, including progabide, baclofen, fengabine and muscimol in both animal models of depression and in clinical trials (Delina-Stula and Vassout, 1978; Morselli et al., 1981; Lloyd *et al.*, 1983, 1987a,b; Singh *et al.*, 1986), although recent clinical reports (e.g. Paykel *et al.*, 1991) have questioned the antidepressant efficacy of such drugs.

A.4 α_2-Adrenergic Receptors

In a functional probe used in the project described in this thesis, chronic treatment with PLZ and N^2AcPLZ attenuated the motor suppressive effects of clonidine, suggesting a decreased α_2-receptor sensitivity. A diminished sensitivity of α_2-adrenergic autoreceptors has been found in other studies of antidepressant treatment and it has been suggested that these changes may precede the decrease in β-adrenoceptors that are thought to be correlated with antidepressant mechanisms (Spyraki and Fibiger, 1980; Reisine *et al.*, 1982; Greenshaw *et al.*, 1988). Activation of α_2-adrenergic receptors on terminals of 5-HT-containing neurones inhibits 5-HT release, and Mongeau *et al.* (1994) have reported that long-term administration of the MAO-A inhibitor befloxatone results in a desensitization of α_2-adrenergic receptors on terminals of 5-HT-containing neurones. The similar effects of PLZ and N^2AcPLZ on α_2-adrenergic receptors further suggest that N^2AcPLZ may be worthy of further investigation as a potential antidepressant.

B. STUDIES OF HYDROXYLATION AS A PATHWAY OF PLZ METABOLISM

B.1 Analysis of *p*-Hydroxy-PLZ

A series of studies was conducted to investigate the possibility of hydroxylation as a metabolic route for PLZ. Indirect evidence of this was found when pretreatment of rats with iprindole followed by PLZ led to higher levels of PLZ and PEA at 3 h compared to levels in rats which received vehicle pretreatment and PLZ. The blocking effect of iprindole on ring hydroxylation is certainly well-documented (Freeman and Sulser, 1972; Fuller and Hemrick-Luecke, 1980; Steranka, 1982), but this drug may have other complex metabolic effects (Hegadoren *et al.*, 1991). More direct evidence was acquired when a peak co-chromatographing with authentic *p*-OH-PLZ was identified in a series of human patients who received PLZ treatment for their psychiatric condition. As mentioned in the Results section, it has not yet been possible to unequivocally demonstrate, on the basis of mass spectral evidence, that this peak is derivatized *p*-OH-PLZ, although GC and MS evidence certainly lends strong support to this contention. Another problem with investigating this metabolite is its apparent instability, as reported in this thesis.

B.2 Effects of *p*-OH-PLZ on Uptake and Release of Neurotransmitter Amines

A series of uptake and release studies showed that *p*-OH-PLZ stimulated the release of ^{3}H-NA, DA and 5-HT from striatal or hypothalamic slices and inhibited their uptake into such slices. As expected from studies on the structurally related amines PEA and *p*-TA (Raiteri *et al.*, 1977), hydroxylation of PLZ resulted in an increased effect on both inhibition of uptake and stimulation of release of such amines. Scarr *et al.* (1991) showed that *p*-OH-PLZ is also a relatively potent inhibitor of MAO, although slightly weaker than PLZ in this regard. As indicated by Dyck (1983), high levels of PLZ are attained in brains acutely after injection, and it will be of interest in future to measure levels of *p*-OH-PLZ in brains of PLZ-treated rats. The assay developed in the present thesis is not suitable for such analysis because of the presence of an interfering peak in rat brain, and a GC-MS assay may have to be utilized in future.

If *p*-OH-PLZ is being formed in humans and rats, the present results suggest that it may make a significant contribution to the overall pharmacological profile of the parent drug. Ring hydroxylation of drugs is often mediated by cytochrome P450 2D6, an enzyme which is inhibited by a number of drugs which may be coadministerred with PLZ (Brosen, 1993; Baker *et al.*, 1994), and thus the possibility exists that there may be important drug-drug metabolic interactions between PLZ and other drugs because of the existence of this route of metabolism.

C. EFFECTS OF PLZ IN HUMAN SUBJECTS

PLZ treatment resulted in a marked clinical improvement in 8 of 13 subjects who presented with anxiety or affective disorders in the present study. In 5 of the 13 patients, no benefit was seen or only a mild improvement or worsening was noted. The treatment group, it should be noted, was characterized in many cases by multiple diagnoses and many of the patients had treatment failures to other medication trials. They were often referred for inclusion in this study because of resistance to other medication trials. Because of the small number of patients investigated, in this thesis there was no comparison of the neurochemical actions of PLZ in those patients who responded with those who were nonresponders. This study was designed to only be a relatively comprehensive neurochemical investigation of the effects of PLZ in a group of depressed patients. Future studies with a larger number of samples and in which the neurochemical results would be compared with clinical measures, as monitored with rating scales for depression and anxiety, would be of great interest.

The urinary levels of PEA, TA, T and 5-HT were dramatically increased following PLZ treatment, in agreement with a previous study from our laboratory (Baker *et al.*, 1985) in which the levels of these amines were investigated in urine samples from dysthymic disorder patients treated with PLZ (levels of the acid metabolites were not investigated in this previous study). Other workers have also found marked increases in urinary excretion of PEA and T after chronic administration of non-selective MAOIs. Dewhurst (1968) reported a dramatic increase in urinary levels of T in patients taking nialamide, and Bieck *et al.* (1984) reported large increases in urinary excretion of PEA and T in subjects treated with TCP. In the present study, the effects of PLZ were most marked on the excretion of PEA and T of the amines investigated, similar to the finding of Baker *et al.* (1985) in dysthymic disorder patients. The increases in urinary levels of the trace amines are presumably due to inhibition of MAO but the metabolism of PLZ to PEA (Baker *et al.*, 1982; Dyck *et al.*, 1985) and possibly also to *p*-TA may also contribute to the increased levels of those two amines.

Urinary levels of the NA metabolites NME and MHPG were increased and decreased, respectively, during treatment with PLZ; a similar pattern was observed with the dopamine metabolites 3-MT and HVA. Such patterns would be expected with a drug that is an inhibitor of MAO, and effects similar to the ones observed here for MHPG and NME have been noted by Bieck *et al.* (1984) and Antonin *et al.*

(1985) in their studies on the effects of MAOIs TCP and brofaromine (a selective, reversible MAO-A inhibitor) on urinary excretion of these metabolites. The 5-hydroxytryptamine (5-HT) metabolite 5-HIAA was not significantly decreased in the present study, and it is of interest that Antonin *et al.* (1985) did not find a decrease in urinary excretion of 5-HIAA at a dose of brofaromine that caused decreased excretion of MHPG (both 5-HIAA and MHPG are metabolites formed by the action of MAO-A on their parent amines). Brofaromine is also a relatively potent inhibitor of 5-HT uptake (Waldmeier, 1993), which may counteract any decrease in 5-HIAA produced by inhibition of MAO-A, but PLZ and N^2AcPLZ are considerably weaker inhibitors of 5-HT uptake than is brofaromine. There was an elevation in levels of *m*-TA and a decrease in its acid metabolite *m*-OH-PAA in the study reported here, with both effects expected as a result of inhibition of MAO by PLZ.

PLZ is both metabolized by and inhibits MAO (Clineschmidt and Horita, 1969a,b). As well as being endogenous compounds, PEA, PAA and *p*-OH-PAA are metabolites of PLZ (Clineschmidt and Horita, 1969a,b; Baker *et al.*, 1982; Dyck *et al.*, 1985; Robinson *et al.*, 1985) and *p*-TA is a putative metabolite (Figure 7 in this thesis). Urinary levels of both PEA and *p*-TA were elevated after treatment with PLZ but no significant changes in the levels of PAA or *p*-OH-PAA were noted. The elevation of PEA in PLZ-treated patients may be explained on the basis of decreased PEA metabolism by MAO-B (which is inhibited by PLZ) and by the formation of PEA from PLZ by metabolism at the hydrazine group (Baker *et al.*, 1982; Dyck *et al.*, 1985). The major route of PEA catabolism is via oxidative deamination by MAO-B to form PAA, but in PLZ-treated patients, where this route is inhibited, ring hydroxylation of PEA to form *p*-TA (a metabolic route which has been demonstrated by others -- Boulton *et al.*, 1975; Callaghan *et al.*, 1985) may become important. In addition, *p*-TA may be formed from *p*-OH-PLZ, a proposed metabolite of PLZ.

In the present study a trend towards increased urinary levels of PAA was noted but did not reach statistical significance. If the major route of formation of PAA was through oxidative deamination of PEA, a decrease in urinary levels of PAA would have been expected. However, it has been suggested that much of the PAA in the body comes from routes other than the one involving PEA (Karoum *et al.*, 1984; Moises *et al.*, 1986). The failure of MAOIs to decrease urinary excretion of PAA has been reported in other studies (Karoum *et al.*, 1984, 1985; Baker *et al.*, 1989). In addition, the formation of PAA from PLZ as a metabolite may be masking any decrease in PAA levels resulting from inhibition of MAO. Similarly, *p*-OHPAA has been reported to be a major metabolite of PLZ (Robinson *et al.*, 1985), and this may counteract decreases in levels of this acid metabolite anticipated after inhibition of MAO. This possibility in consistent with the observation that levels of *m*-OHPAA, the acid metabolite of *m*-TA, were decreased by treatment with PLZ. However, Karoum (1985) has reported urinary excretion of *p*-OHPAA in rats was also unaltered by chronic administration of the MAOIs pargyline, clorgyline or deprenyl; at the same doses and time intervals, all three drugs decreased urinary levels of HVA and MHPG.

In summary, a comprehensive study has been conducted on urinary excretion of several important biogenic amines and their metabolites in psychiatric patients before and during treatment with PLZ. Marked increases in the urinary excretion of trace amines (PEA, T, TA), 5-HT, 3-MT and NME were observed along with decreases in the excretion of MHPG and HVA, metabolites of NA and DA, respectively, after administration of PLZ. Although PLZ treatment resulted in decreased urinary levels of *m*-OH-PAA, no such decrease in *p*-OH-PAA levels was observed. No decreases in urinary excretion of the acid metabolites PAA and 5-HIAA were noted. In the cases of PAA and *p*-OH-PAA, the lack of a decrease may be explained in part by the metabolism of PLZ to these acids (Robinson *et al.*, 1985).

SUMMARY

1. *PLZ and N^2AcPLZ were compared with regard to their neurochemical actions after both acute and chronic administration.*

(a) Like PLZ, N^2AcPLZ is an inhibitor of MAO-A and -B *ex vivo* and *in vitro* and results in an increase in brain levels of catecholamines and 5-HT and a decrease in the levels of HVA, DOPAC and 5-HIAA. N^2AcPLZ is somewhat weaker than PLZ in this regard, but is potent enough that it warrants further investigation as an antidepressant in its own right.

(b) While PLZ produced a marked elevation of brain levels of both GABA and ALA, N^2AcPLZ was devoid of this effect, even at doses much higher than employed for PLZ. If, as has been proposed, the antipanic effects of PLZ are due to its action on GABA (Breslow *et al.*, 1989), N^2AcPLZ may be a very useful drug with which to compare PLZ to ascertain the importance of GABAergic actions in PD. Neither drug produced an effect on brain levels of GLY, VAL or LEU.

(c) PLZ, after chronic administration, produced significant decreases in the activity of GAD, GABA-T and ALA-T in rat brain. The activity of GABA-T was reduced more than that of GAD, presumably contributing to the overall increase of GABA levels. The % inhibition of both GABA-T and ALA-T was quite small (32% and 11%, respectively), suggesting that other effects of PLZ in addition to inhibition of these transaminases may be contributing to the increases in brain levels of GABA and ALA.

(d) In acute studies, it was demonstrated that both the GABA-elevating and the ALA-elevating effects of PLZ could be reversed by pretreatment of the rats with MAOIs of two types, namely TCP (a cyclopropylamine) and pargyline (a propargylamine). Neither TCP nor pargyline affected levels of GABA or ALA on their own. These observations provide further evidence that the elevating effects on GABA and ALA are due, at least in part, to the direct interaction of PLZ with MAO.

(e) In a behavioural test using the α_2-adrenergic receptor agonist as a behavioural challenge, it was demonstrated that PLZ and N^2AcPLZ cause down-regulation of α_2-receptors, a characteristic of many other antidepressants. This finding further indicates that N^2AcPLZ should be tested as a potential antidepressant in its own right.

(f) Chronic administration of PLZ or N^2AcPLZ resulted in no changes in K_D or B_{max} values with regard to binding to $GABA_A$ receptors in cortex (measured using ^{3}H-muscimol) or benzodiazepine receptors in cortex or hippocampus (measured using ^{3}H-flunitrazepam). These findings are in agreement with those of several other researchers using other antidepressants.

2. *Studies on hydroxylation of PLZ.*

(a) Indirect evidence for ring hydroxylation of PLZ was provided by studies in which pretreatment with IPR, a known inhibitor of ring hydroxylation, resulted in higher rat brain levels of PLZ than did pretreatment with saline vehicle.

(b) Gas chromatographic analysis indicates a peak that co-chromatographs with *p*-OH-PLZ in urines taken from patients on PLZ, but unequivocal mass spectrometric evidence is still lacking. The GC results suggest that *p*-OH-PLZ is extensively conjugated and that it is relatively unstable upon storage.

(c) Uptake and release experiments in slices from rat hypothalamus or striatum indicate that *p*-OH-PLZ is more potent than PLZ at stimulating release of DA and 5-HT and is more potent than PLZ at inhibiting uptake of DA, NA and 5-HT.

3. *Effects of PLZ on excretion of amines and metabolites in human subjects.*

PLZ administration for 2 and 4 weeks resulted in dramatic increases in urinary levels of the amines PEA, *m*- and *p*-TA, T, 5-HT, 3-MT and NME and decreases in urinary levels of HVA, MHPG and *m*-OH-PAA. Unexpectedly, however, levels of the acid metabolites of 5-HT, PEA and *p*-TA (namely 5-HIAA, PAA and *p*-OH-PAA) did not change significantly from pretreatment levels. It is possible that increases in PAA and *p*-OH-PAA in urine as a result of metabolism of PLZ may mask any decreases in levels of the two metabolites produced by inhibition of MAO. However, a similar lack of effect on urinary levels of PAA and *p*-OH-PAA has also been reported with other MAOIs.

In summary, experiments have been performed which have provided significant novel information about the neurochemistry and metabolism of the MAOI antidepressant/antipanic drug PLZ and about the neurochemical actions of N^2AcPLZ, a MAOI with potential as an antidepressant in its own right.

POSSIBLE FUTURE STUDIES

The findings from this thesis project have suggested some exciting new possibilities for research which should be pursued with PLZ:

1. PLZ and N^2AcPLZ should be compared for their activity in animal models of depression and PD. As indicated in this thesis, N^2AcPLZ may prove to be a useful antidepressant without some of the side-effects of PLZ which seem to be the result of the free hydrazine group in the latter drug. Since both drugs inhibit MAO but only PLZ elevates GABA, a comparison of the antipanic effects should indicate the relative importance of GABA in the antipanic effects of PLZ, a matter of considerable clinical interest.
2. Molecular biology technologies which permit investigation of expression of mRNAs for isoforms of $GABA_A$ receptor subunits should be applied to brain areas following chronic administration of PLZ, particularly since recent reports in the literature suggest that changes in the ratios of such isoforms occur upon administration of benzodiazepines, drugs which also increase GABAergic effects in the brain. No changes in density or affinity of $GABA_A$ receptors were observed in the current study using radioligand binding experiments, but it is possible that chronic administration of PLZ may result

in substitution of one subunit isoform with another; such a substitution could change the characteristics of the drug response without necessarily changing binding capacity. Joint studies on this aspect of $GABA_A$ receptor function are now underway between the Departments of Pharmacology and Psychiatry at the University of Alberta.

3. Techniques utilizing GC-MS, tandem MS, or combined HPLC-MS should be examined as possible means for developing a suitable routine assay for *p*-OH-PLZ. Such sophisticated instrumentation was not available on a routine basis for the present investigation. These techniques could also be combined with administration of stable isotope-labelled *p*-OH-PLZ to rats to determine if this compound is converted to *p*-TA and/or *p*-OH-PAA.

4. Further detailed studies to determine the metabolite(s) contributing to the GABA-elevating actions of PLZ are warranted since such metabolites may be useful drugs in their own right.

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APPENDIX I

Publications arising from the results described in this thesis

PAPERS IN REFEREED JOURNALS AND BOOK CHAPTERS:

McKenna K.F., Baker G.B., Coutts R.T., Rauw G., Mozayani A. and Danielson J.T. (1990) Recent studies on the MAO inhibitor phenelzine and its possible metabolites. *J. Neural Transm.,* **32(Suppl):** 113-118.

McKenna K.F., Baker G.B. and Coutts R.T. (1991) N^2-Acetylphenelzine: effects on rat brain GABA, alanine and biogenic amines. *Naunyn Schmied. Arch. Pharmacol.,* **343:** 478-482.

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ABSTRACTS:

McKenna K.F., Mozayani D., Coutts R.T., Danielson T.J. and Baker G.B. (1989) N^2-acetylphenelzine, an active metabolite of phenelzine: neurochemical studies. *Proc. 12th Meet. Int. Soc. Neurochem.,* Algarve, Portugal, J. Neurochem. **52 (Suppl.):** S153D.

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